Studies of Gliogenesis in the Central Nervous System of Zebrafish (*Danio rerio*)

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A thesis submitted to the University of London as partial fulfilment for the degree of Doctor Philosophy (Ph.D.)

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July 2005

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ABSTRACT

Oligodendrocytes (the myelinating cells of the central nervous system, CNS) and astrocytes (a heterogeneous class of cells with several different functions) constitute the two major classes of glial cells in the CNS. Both are found widely distributed in the white and grey matter of the adult mammalian brain. Glial cells, like neurons, develop from neuroepithelial precursor (stem) cells in the ventricular zone (VZ) of the brain and spinal cord. In the spinal cord, most oligodendrocyte progenitors (OLPs) originate from a restricted region of the neuroepithelium close to the floor plate (pMN domain) that earlier generates motor neurons, suggesting a common precursor for these cells. OLPs migrate out from this region throughout the CNS where they proliferate and differentiate, differentiating and creating myelin mainly in the white matter. Astrocytes are thought to arise from multiple locations along the dorsal-ventral axis of the VZ, distributing into both grey and white matter.

Amongst other characteristics, the embryonic transparency, high fecundity and possibility of performing a large-scale mutant screens make zebrafish an attractive model for studying gene function and identifying new components of the genetic pathways regulating developmental processes. The primary goal of this project was to investigate the possibility of using zebrafish as a model for glial development in higher vertebrates including mammals, focusing primarily on oligodendrocyte development. I have examined the development of oligodendrocytes in immature and adult zebrafish spinal cord by investigating the expression of the zebrafish homologues of oligodendrocytes lineage: *olig1*, *olig2*, *sox10*, *pdgfrα*, *nk2.2*, and myelin markers: *mbp*, *plp* and p_0. From these investigations I show that oligodendrocyte development in zebrafish is very similar to that of higher vertebrates. The characterization of these oligodendrocyte lineage markers, establishes a baseline to screen for mutants with defects in myelin. One mutant isolated in a pilot screen, *otter*, was characterized and demonstrated a defect in oligodendrocyte maturation.

One difference between zebrafish and higher vertebrates is the absence of *pdgfrα* expression in OLPs of zebrafish. I have used a transient transgenic approach to determine whether the *cis* human regulatory function of the elements that direct expression of the human PDGFRα gene in OLPs and non-CNS tissues are conserved in fish. Two vectors were used: Human PDGFRα 2.2kb-*lacZ* fragment (2.2kb-*lacZ*) containing non-CNS and human PDGFRα 110kb BAC containing both non-CNS and OLP specific cis-regulatory elements. 2.2kb-*lacZ* established the correct spatio-temporal expression in mesoderm and neural crest derived tissues, similar to the endogenous expression in zebrafish and the expression described in transgenic mouse (Zhang et al., 1998). Similarly, the 110kb BAC transgene gave a similar expression to non-CNS tissues, but it was also upregulated in scattered cells in the zebrafish CNS. However, due to technique limitations it was not possible to demonstrate that these cells were oligodendrocytes.

Supplementary work included in this thesis studied homologues of genes known to be involved in astrocyte development in mammals including: fibroblast growth factor receptor 3 (*fgfr3*), glutamine synthetase (*gs*) and glial fibrillary acid protein (*gfap*). The patterns of expression were investigated in developing and adult zebrafish and *Xenopus*.

ACKNOWLEDGEMENTS

Writing this page (and, of course, the rest of this thesis) would not have been possible without the support of many and varied people, both before and during my time at the Wolfson Institute.

First, I thank my parents: Ana and Custodio. They have been more supportive than is it possible to express here. This thesis is dedicated to their love and sacrifice. It goes without saying that this is extended to my sisters: Dolores, Carmen, María José and Custi, and the little ones, María and Ismael.

I also thank my supervisors, Prof. Bill Richardson and Dr. Hazel Smith, for giving me the opportunity to do this PhD and their sustained efforts throughout my period of study.

Thanks also to past and present members of the Lab. Students, post-docs, technicians, from many different backgrounds and nationalities, most, if not all have contributed in some way. There are so many that if I try to list them all I am afraid that I might forget some names, but I will never forget the immense help and support they have given me. One person I should mention is Dr Huiliang Li, my zebrafish colleague and good friend, who helped injection techniques and much more. I wish you all health and happiness.

Special mention goes to Professor Steve Wilson and his group, especially Dr. Florencia Casanova and Dr. Tom Hawkins, for their excellent zebrafish facility, Tim Geach for the *Xenopus* embryos and Natasha Kazakova for the mutant embryos and electron microscopy. Also, thanks to the Wolfson Institute IT support team.

On a personal level, I am extremely grateful to Kath Adley for her unswerving friendship and unflinching response to initial drafts of this thesis. Together with Robin Williams we had times (and lunches) always to be remembered. Thanks also to Juanma Funes for support and a place to stay.

Dr. Milagros Gallo, Prof. Agustina Garcia, Dr. Sara Patel, Dr. Victoria Velasco and Ester Toms have been personal and professional role models for many years, I thank them for the direction and motivation they have provided me. This also from my friends from all around the world; in particular, for their understanding of being neglected: Monica, Tahira, Erwann, Araceli, Barbara, Lola, Pepe, Helen, Francois, Lisi and Alcyr, Teresa. Mely, Maria, Nieves, Tusti and many more.

Finally, the most important person in my life: John. Without him this thesis would not have been completed (literally!). His constant love and encouragement have been precious during the good and the bad times during these years.

The work in this thesis was supported by a CASE studentship from BBSRC and GlaxoSmithKline plc.

Esta tesis esta dedicada a mis padres, por su amor, sacrificio y apoyo.

CONTENTS

LIST OF FIGURES and TABLES

ABBREVIATIONS

A-P	Anterior-Posterior axis
BAC	Bacterial artificial chromosome
BCIP	5-bromo-4-chloro-3-inolyl-phosphate
β-gal	Beta Galactosidase
bHLH	basic Helix Loop Helix
BMP	Bone morphogenetic protein
Bp	Base pair
BrdU	5-bromo-2'-deoxyuridine
cDNA	Complementary deoxyribonucleic acid
CNS	Central nervous system
CNP	2',3'-cyclic-nucleotide 3'-phosphodiesterase
DAPI	4',6-diamidino-2phenylindole
DCTP	2'-deoxy-cytidine-5'-triphosphate
DEPC	Diethylpyrocarbonate
DIG	Digoxygenin
Dll	Distalless
DM-20	26.5kDA protein isoform encoded by *Plp* gene
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
dpf	Days post fertilization
dNTP	Deoxynucleoside triphosphate
DTT	Dithiothreitol
D-V	Dorsoventral axis
E	Embryonic day
EDTA	Ethylene diamine tetra-acetic acid (disodium salt)
ESTs	Expressed sequence tag
FITC	Fluorescein isothiocyanate (green fluorophore)
FGF s	Fibroblast growth factor
FGFR3	Fibroblast growth factor receptor 3
FP	Floor plate
g	Gram

gDNA	Genomic deoxyribonucleic acid
GFP	Green fluorescence protein
GFAP	Glial fibrillary acidic protein
GC	Galactocerebroside
GCL	Ganglion cell layer
GLAST	Glutathione transferase
GS	Glutamine synthetase
GM	Ganglionic eminence
GRP	Glial restricted precursors
hpf	Hours post fertilization
HSV	Herpes simplex virus
IMF	Immuno fluorescence
INL	Inner nuclear layer
IPL	Intraperiod line
ISH	*In situ* hybridization
kb	Kilobase pair
Kda	KiloDalton
l	Litre
LB	Luria- Bertani
LG	Linkage Group
LGE	Lateral ganglionic eminence
MDL	Major dense lines
MGE	Medial ganglionic eminence
MBP	Myelin basic protein
MN	Motor neuron
mg	milligram
ml	Millilitre
mRNA	Messenger ribonucleic acid
NC	Notochord
neo	Neomycin resistance
Ngn2	Neurogen 2
ng	Nanogram
NBT	Nitroblue tetrazolium salt
OD^n	Optical density at a wavelength of 'n' nm

OLP	Oligodendrocyte precursors
OMIM	Online Mendelian Inheritance in Man entry
ONL	outer nuclear layer
P	Postnatal age (days after birth)
p	Progenitor domain
P0	Myelin protein zero
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDGFRα	Platelet-derived growth factor receptor alfa
PDGF-A	Platelet derived growth factor ligand A
PDL	Poly-D-lysine
PH	Prolyl hydroxylase
PNS	Peripheral nervous system
PLP	Proteolipid protein
PTU	1-phenyl-2-thiourea
RNA	Ribonucleic acid
RNase	Ribonuclease
SDS	Sodium dodecyl sulphate
Shh	Sonic Hedgehog
SVZ	Sub-ventricular zone
tww	*tiggy-winkle hedgehog*
UCL	University College London
UV	Ultraviolet
VZ	Ventricular zone
WT	Wild type
YAC	Yeast Artificial Chromosome
μg	microgram
μl	microlitre
μm	micrometer

PREFACE

This thesis is an investigation of the development of glial cells - oligodendrocytes and astrocytes - in the central nervous system (CNS) of the zebrafish *(Danio rerio)*. *Chapter 1*, the introduction, begins with a brief description of general vertebrate nervous system development. Then, a short resumé of what we know up to the present date about oligodendrocyte and astrocyte development from studies carried out mainly in mammals and birds. It ends with an overview of the advantages of zebrafish as a model and a statement of the aims of the project.

Following a description of the experimental methods in *Chapter 2*, *Chapters 3* and *4* examine in detail a number of experiments concerning the development of oligodendrocytes in immature and adult zebrafish CNS. These studies clarify the expression of mammalian homologue genes involved in oligodendrocyte development. The glial development of the amphibian *Xenopus laevis,* is comparatively analysed in a similar manner. Part of this chapter has been submitted for publication (Mora et al., 2005). *Chapter* 4 also reports on experiments revealing the expression of glia lineage markers in the *otter* (*ott:*) fish line, in a search for defects in glia development. Part of this data is to be submitted for publication (Kazakova et al 2005).

Further insight into the differences between PDGFRα gene in zebrafish and mammals is given in *Chapter 5*. In these studies a transgenic approach and detailed descriptive transient expression analyses were used. DNA construct containing the human *PDGFRα* gene driving expression of OLPs in CNS in mouse was injected into the one cell stage in zebrafish.

Chapter 6 describes astrocyte development in immature and adult zebrafish and *Xenopus* CNS from analysis of the homologues of genes involved in astrocyte development in mammals. *Chapter 7* concludes the thesis with an overall summary and a discussion of the importance and impact of this research to future experiments.

As an aside on notation, the spinal cord outline is defined in the figures by dashed line.

Some *in situ* hybridization and immunostaining were detected by fluorescence. To avoid confusion, the labelling of either *in situ* hybridization (ISH) or antigen (IMF) is stated in the appropriate figure legends. Further, to distinguish species-specific genes; zebrafish genes are in lower case and italicised, e.g *pdgfrα*. *Xenopus* genes are written with the first letter in uppercase, further letters in lower case and italicised, e.g *Pdgfrα*. Human genes are written in upper case and italics, e.g *PDGFRα*.

Chapter 1

General Introduction

1.1 The Vertebrate Central Nervous System: An Overview

The vertebrate central nervous system (CNS) is the most fascinating and most complicated of organs. It consists of two major classes of neural cells: neurons and glial cells. Neurons are the electrically excitable cells receiving and transmitting information to and from other cells. Glial cells are specialized cells that help to construct the nervous system during embryonic development as well as maintaining its daily functions in adult life. The vast majority of cells in the CNS are glial cells and not neurons. It is estimated that glial cells occupy half of the human brain volume and outnumber neurons by 10 to 1 in many parts of the CNS. Intriguingly, the different neuronal and glial cell types are originally derived from a simple, apparently homogeneous population of neuroectodermal cells that constitute the embryonic neural tube. Only after these cells go through a combination of proliferation, migration and differentiation in the developing CNS are functionally distinct brain structures generated. How does the apparently homogeneous population of neuroectodermal cells that constitute the neural tube differentiate and give rise to the multiple cell types and complex structures of the mature brain? This is one of the greatest challenges in developmental biology. Many kinds of experimental systems have been used to understand the development of neuroectodermal cells and the structures to which they eventually give rise. Such methodologies include experiments with very simple models like yeast cells, invertebrate models such as D*rosophila* melanogaster and *Caenorhabditis* elegans as well as vertebrate models such as *Xenopus laevis*, chick, mouse and zebrafish, *Danio rerio*, which is increasingly popular due to its transparency and the ease with which mutations can be made in developmental genes. For this and other reasons, the zebrafish model has been chosen for the studies contained in this Thesis. However, the vast majority of studies of vertebrate cell development and embryogenesis have been conducted in birds and mice, and they are mainly described in this introduction.

Three major types of glial cells are recognised in the CNS: oligodendrocytes, astrocytes and microglia. Oligodendrocytes synthesise and maintain the myelin sheaths that wrap around axons, providing insulation as well as facilitating saltatory (jumping) conduction of action potentials a reviewed by (Pfeiffer et al., 1993). Astrocytes are the most numerous of glial cells, performing a number of distinct tasks. They maintain the extracellular ionic

environment for neuronal function, balancing the pH, recycling neurotransmitters, storing energy and regulating vasculature. They are also responsible for a multitude of trophic functions (Powell and Geller, 1999; Powell et al., 1999; Vernadakis, 1996) and recent findings indicate that astrocytes also communicate with neurons at synapses (Fields and Stevens-Graham, 2002). Microglial cells constitute the least numerous of the three classes of glia in the vertebrate CNS. They are unrelated to the other CNS cell types, being specialized macrophages that protect neurons (Kaur et al., 2001). Oligodendrocytes and astrocytes derive from the same neuroepithelial cells that give rise to neurons.

1.2 Early development: neurulation and differentiation of the neural tube.

The vertebrate CNS - including the brain, spinal cord and retina of the eye - arises from the embryonic ectoderm by a process known as neurulation. Following an inductive signal from the underlying mesoderm, a broad region of the ectoderm thickens and forms the *neural plate* (Figure 1.1A). Through neurulation, the neural plate folds along its central axis creating a *neural groove* lined on each side by a *neural fold* (Fig 1.1B). The neural folds then fuse together converting the groove into a cylindrical structure termed the *neural tube* (Figure 1.1C), beginning in the hindbrain region of the embryo and moving distally. These stages, which overlap temporally, have been studied mostly in amphibians such as *Xenopus laevis* and also the chick.

Once neurulation is completed, the walls of the neural tube comprise a single layer of cells known as the neuroepithelium (neuroectoderm). This apparently homogenous population of cells goes on to produce the wide variety of neuronal and glial cell types found in the mature CNS. As neural development proceeds, the cephalic (head) region of the neural tube forms three primary vesicles: forebrain (prosencephalon), midbrain (mesencephalon) and hindbrain (rhombencephalon). Subsequently, the prosencephalon subdivides into telencephalon and diencephalon and the rhombencephalon subdivides into the metencephalon and myelencephalon. The caudal region of the neural tube does not subdivide but does increase in size and undergoes further differentiation to form the spinal cord.

In zebrafish, neurulation is completed by 17 hours postfertilization (17hpf). The basis processes of neurulation are the same as tetrapods except that the neural tube starts as a solid cylinder of cells and the lumen forms subsequently by death of the cells at the centre.

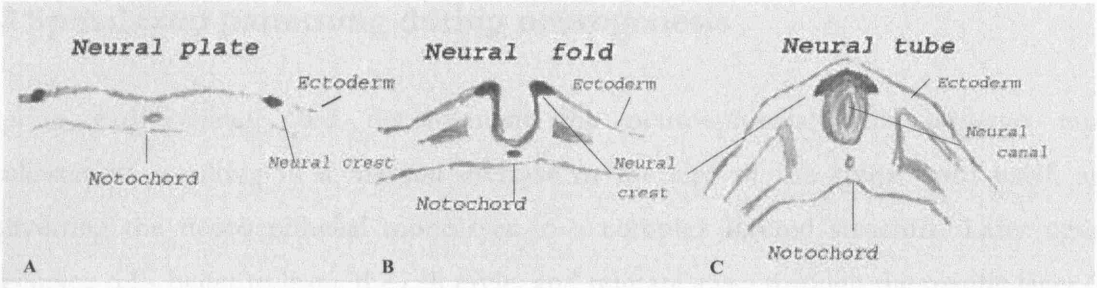


Figure 1.1 Stages in the development of the spinal cord. During early development the CNS consists of the neural tube arising from the ectoderm, while the notochord and somites arise from mesoderm. **A.** Notochord induces its overlying ectoderm to become the neuroectoderm and develops into the **neural plate**. **B**. The neural plate folds along its central axis to form a **neural groove**. **C.** The neural groove fuses and pinches off to become the **neural tube**. The three brain vesicles, forebrain, midbrain and hindbrain are formed at the rostral end of the neural tube. The spinal cord is formed at the caudal end. Adapted from Tanabe and Jessel, 1996.

Neuroepithelial cells within the embryonic neural tube require external positional information in order to know their precise location and differentiate accordingly. The diffusible factors that provide this information control the fate of early neuroepithelial cells and determine the type of neuron or glial cells into which they develop. Cell fate in the neural tube depends on two signalling systems along the anterior-posterior (A-P) and dorso-ventral (D-V) axes of the neural tube that establish a grid-like set of positional cues (Lumsden and Krumlauf, 1996; Pituello, 1997). Signalling along the A-P axis determines the main subdivisions of the CNS: forebrain, midbrain, hindbrain and spinal cord (Lumsden and Krumlauf, 1996). The D-V signalling system creates cell type diversity within each of the A-P subdivisions (Pituello, 1997). Graded concentrations of signaling molecules such as retinoic acid, fibroblast growth factors (FGFs), hedgehogs, Wnts and bone morphogenetic proteints (BMPs) have been shown to be involved in spinal cord patterning (Lumsden and Krumlauf, 1996). The patterning mechanisms appears to be highly conserved between species, for example in rat, mouse and chicken (Lumsden and Krumlauf, 1996; Jessell, 2000).

The spinal cord has been the most extensively studied and described region of the vertebrate CNS, as it is the most highly conserved region. It has also been used to study the molecular mechanisms that control cell fate in the CNS as described in the following section and is the main structure in which the experiments of this Thesis were performed.

1.3 Spinal cord patterning during neurogenesis

During early spinal cord development the neuroepithelial cells undergo rapid proliferation, resulting in a marked increase in the size of the spinal cord itself, and converting the neuroepithelial monolayer to a complex layered structure. Later neural precursor cells begin to leave the cell cycle, and migrate away through the mantle layer (or intermediate zone) to their final positions. Here they differentiate into the various types of neurons and glial cells found in the mature cord. The distinct neuronal and glial cells types are generated from discrete progenitor domains located along the D-V axis of the neuroepithelial layer (Jessell, 2000; Briscoe et al., 2001). These progenitor domains are characterized by the controlled expression of a group of transcription factor genes established by concentration gradients of secreted protein signals. One such signal is Sonic hedgehog (Shh), which is provided initially by axial mesodermal cells of the notochord that underlie the neural tube, and subsequently by cells of the floor plate (Briscoe et al., 2000; Ericson et al., 1997; Briscoe and Ericson, 1999; Briscoe and Ericson, 2001; Echelard et al., 1993; Roelink et al., 1995). Other protein signals including BMP2 and BMP4 originate from the roof plate at the dorsal midline (review by (Lee and Jessell, 1999; Lee et al., 1998). Both Shh and BMPs are thought to diffuse away from their source to form opposing concentration gradients along the D-V axis (Briscoe et al., 2000; Tanabe and Jessell, 1996) (See Figure 1.2)

The combined expression of a set of transcription factors divides the spinal cord neuroepithelium into different subdomains along the D-V axis. Five progenitor domains have been described so far within the ventral spinal cord alone. From dorsal to ventral they are known as: p0, p1, p2, pMN and p3 domains which generate V0, V1, V2, somatic motor neurons (sMN) and V3 interneurons respectively (Briscoe and Ericson, 2001; Gowan et al., 2001; Jessell, 2000) (Figure 1.2). These transcription factors have been

categorized into class I and class II proteins, based on their regulation by Shh (Briscoe et al., 2000).

The expression of Class I proteins, including Pax7, Dbx1, Dbx2, Irx3, and Pax6 are repressed by Shh signalling (Briscoe et al., 2000; Briscoe and Ericson, 2001). In contrast, the expression of Class II proteins such as Nkx6.2, Nkx6.1, Olig2, Nkx2.2 and NKx6.2 depends on Shh signalling (Briscoe and Ericson, 2001; Briscoe et al., 2000; Novitch et al., 2001; Mizuguchi et al., 2001; Vallstedt et al., 2001). All these encoded proteins are homeodomain transcription factors (Jessell, 2000), except for Olig2 which is a basic helix loop helix transcription factor (bHLH) (Zhou et al., 2000; Lu et al., 2000; Takebayashi et al., 2000).

There are also cross-repressive interactions among these transcription factors (Briscoe et al., 2000). These interactions appear to have three main roles. First, they establish the initial D-V domains of expression of Class I and Class II proteins. Second, they ensure

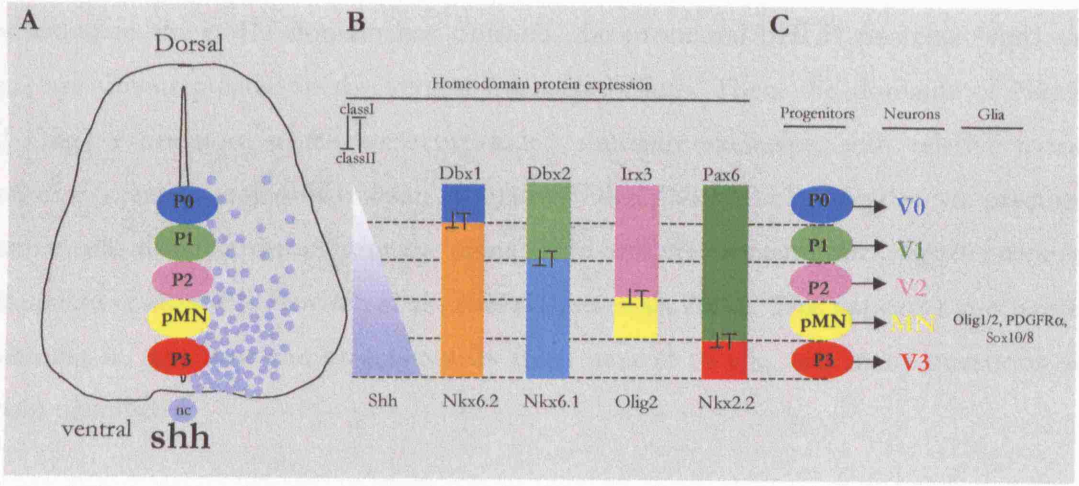


Figure 1.2 Cell fate specification in the vertebrate spinal cord. A. Shh dependent D-V patterning in the neural tube. The floor plate (fp) cells and the underlying notochord cells (nc) produce the secreted molecule Shh. **B.** Ventral sources of Shh set a gradient, inducing Class I proteins (Dbx1, Dbx2, Irx3 and Pax6) or repressing Class II proteins (Nkx6.2, Nkx6.1, Olig2 and Nkx2.2) defining the progenitor domains p0, p1, p2, pMN (motor neuron) and p3 in the ventral neural tube. **C.** These domains will give rise to different cell types; ventral interneurons cell types v0, v1, v2, v3 and MN (somatic motorneurons). pMN domain will also give rise to oligodendrocytes identified by the expression of Olig1, Olig2, Sox10 and platelet derived growth factor receptor α (PDGFRα). Adapted from Jessell, 2000.

the sharpening and maintenance of the different domain boundaries. Third, the combinatorial expression of these two types of proteins regulate downstream genes to specify the neuronal subtype within each boundary (Briscoe and Ericson, 2001; Briscoe et al., 2000).

It is known that neurogenesis occurs before gliogenesis (oligodendrocytes and astrocytes formation). It is now recognized that glial precursors are formed before birth; several studies using markers of immature oligodendrocytes have shown that they are first formed in the ventral neural tube around E12.5, which is however still after ventral neurons. A number of studies using early glial precursor markers as well have shown that glial specification is indeed after that of neurons (Lu et al., 2000; Yu et al., 1994; Hall et al., 1996; Zhou et al., 2000). What it is not known is how this temporal decision, - the switch from neurons to glial cells - is made. Several reports indicate that neurogenic bHLH transcription factors promote neuronal fate whilst simultaneously repressing glial fate; that is, there is a molecular switch between neuronal and glial fate (Lu et al., 2002; Zhou and Anderson, 2002; Rowitch et al., 2002). During midgestation when motor neuron production in the pMN domain has finished, the proneural bHLH proteins Ngn1 and Ngn2 are downregulated in the ventral neuroepithelium. Then, the domains of Nkx2.2 and Olig2 expression, which were previously mutually exclusive, shift relative to one another to create a region of overlap. Migratory Olig2/Nkx2.2 oligodendrocyte precursor positive cells disperse throughout the spinal cord and differentiate into oligodendrocytes (Mizuguchi et al., 2001; Novitch et al., 2001; Zhou et al., 2001). This appears a mechanism explaining in part how the switch occurs from neuron to glia, but many questions still remain unsolved.

The zebrafish spinal cord has features similar to the neural tube of rodent and chick embryos. A pattern along the D-V axis can be described with distinct cell types located in specific D-V domains. At 24hpf, the spinal cord consists solely of non-neuronal floor plate cells, which are located at the ventral midline of the cord, neuroepithelial cells surrounding the central lumen and roof plate cells located at the dorsal midline. Neuroepithelial cells are proliferative neural precursors that give rise to neurons and glia. As with to other higher vertebrates, the zebrafish spinal cord contains primary and

secondary neurons (Westerfield et al., 1986; Lewis and Eisen, 2003). Primary neurons have large cell bodies, are few in number, are born during gastrulation and have cell specific axonal projections (Myers et al., 1986). Secondary neurons are smaller, more numerous, born later (Appel and Chitnis, 2002; Myers et al., 1986) and have finer axons than primary neurons. Primary neurons consist of sensory, inter- and motor neurons while secondary neurons consist only of interneurons and motor neurons. Roughly speaking sensory neurons occupy the most dorsal position, motor neurons occupy relatively ventral positions and interneurons are located at intermediate positions. The secondary neurons are probably more similar than primary neurons to the spinal neurons in amniote vertebrates, but less is known about them than about primary neurons in zebrafish.

Cell fate specification has not been thoroughly analysed yet in zebrafish spinal cord; however, the available data are consistent with the idea that signals that act at a distance from dorsal and ventral sources (Bmp and Shh signalling) pattern the zebrafish spinal cord in a manner analogous to chick and rodent (Appel et al., 2001; Lewis and Eisen, 2003).

As in other vertebrates, the zebrafish spinal cord contains glial cells in addition to neurons: oligodendrocytes (Tomizawa et al., 2000a; Brosamle and Halpern, 2002; Park et al., 2002) astrocytes (Kawai et al., 2001) and radial glia (Tomizawa et al., 2000b). A further description of these cells in the zebrafish is given in Chapters 3, 4 and 6.

The studies reported in this Thesis focus on the characterization and developmental origins of glial cells (oligodendrocytes and astrocytes) from the neuroepithelium, mainly in the spinal cord.

1.4 Glial cell lineage in the CNS

Glia were named and identified by Rudolf Virchow in 1856. He described them as a connective substance formed in the brain and spinal cord in which the nervous system elements are embedded. Later, in 1885, Golgi observed that glia contacted both blood

vessels and nerve cells and in 1889 he suggested that radial glial cells acted as guides for migrating neurons during development.

Unlike neurons, glia does not have chemical synapses, nor do they communicate via action potentials or neurotransmitters. Furthermore, they do not lose the ability to multiply after birth and are able to invade damaged regions and clear away necrotic material. Like neurons, glia shows many different structural forms and form many subtypes. There are three major types of glia: oligodendroglia, astroglia and microglia, each type having an important role in the function of the nervous system.

1.4.1 Oligodendroglia cells

Oligodendroglia were originally identified and named by Rio Hortega 1921. He visualized them using metallic impregnation techniques. Literally, they are neuroglial cells with a few (=oligo) dendritic processes radiating from the cell body. Oligodendrocytes are found mainly in the white matter, but also in the grey matter. Their primary role is to synthesize and maintain a specialised structure, the myelin sheath, which wraps around axons facilitating transmission of nerve impulses by saltatory conduction (Lee, 2001; Baumann and Pham-Dinh, 2001).

More recently, studies have shown that oligodendrocytes also contribute to neuronal survival and development as well as neuronal transmission and synaptic activity (Sanchez et al., 1996; Sortwell et al., 2000; Pfrieger and Barres, 1997).

In the peripheral nervous system (PNS), there is another type of glial cell known as Schwann cells. They have a similar function to oligodendrocytes, providing myelination to axons in the PNS. Peripheral and central myelin is not laid down in the same way, but the end result or function is much the same. Schwann cells also have phagocytotic activity and clear cellular debris that allows for re-growth of PNS neurons. Another difference to oligodendrocytes is that Schwann cells only contribute to one internode where a single oligodendrocyte can myelinate up to fifty axonal segments of the surrounding neurons in close proximity (that is, within the same region of the CNS) (Butt and Ransom, 1989).

Understanding the genes involved in the early specification and development of these myelin-forming cells during embryogenesis could be crucial to the development of therapies for degenerative diseases such as multiple sclerosis, which result of oligodendrocyte death.

1.4.1.1. Ventral origin of oligodendrocytes and their relationship with motor neurons in the spinal cord

Oligodendrocytes are widely distributed throughout the CNS but their origins remain a source of debate. Since they are widely dispersed throughout the adult CNS, it might be reasonable to suppose that they were produced all throughout the neuroepithelium. It was thought that both oligodendrocytes and astrocytes differentiate directly from embryonic radial glial cells and therefore share a common precursor in the spinal cord (Hirano and Goldman, 1988; Choi and Kim, 1985). Radial glia are another type of glial cells that extends from the ventricular to the pial surface. They were thought to give structural support to the CNS and to provide a surface for neuronal migration. However, lately, they have been shown to play a central role as neural precursors, in production of neurons, astrocytes and oligodendrocytes (Parnavelas and Nadarajah, 2001; Malatesta et al., 2000; Miyata et al., 2001; Rakic, 2003). Le Vine and Goldman (1988) thought that oligodendrocytes could arise from radial migration from the germinal ventricular zone (VZ) of the CNS. Later, many different lines of evidence suggested that most of the earliest oligodendrocyte precursors in the spinal cord originate from a discrete region in the VZ located near the floor plate (Ono et al., 1995; Timsit et al., 1995; Pringle et al., 1996; Pringle and Richardson, 1993). It is now known that the majority of oligodendrocytes are derived from the ventral VZ but that a minority of spinal cord oligodendrocytes are generated from more dorsal domains (Cai et al., 2005; Fogarty et al., 2005; Vallstedt et al., 2005). The ventral, major source generated progenitor cells that migrate widely throughout the cord before differentiating into myelin forming oligodendrocytes.

Studies of spatio-temporal gene expression patterns in the embryonic mouse and chick spinal cord show that most oligodendrocyte progenitors (OLPs) arise from the same specific region in the ventral neuroepithelium that earlier produced motor neurons the

pMN domain suggesting a common origin for motor neurons and oligodendrocytes (Lu et al., 2000; Tekki-Kessaris et al., 2001; Woodruff et al., 2001; Briscoe and Ericson, 2001). For example, PDGFRα positive OLPs begin to appear in mouse embryos at E12.5 (Calver et al., 1998) in pMN after the production of motor neurons (E8.5- E12). Olig bHLH transcription factors (Olig1 and Olig2) are expressed in myelinating oligodendrocytes and migratory OLPs as well as in neuroepithelial precursors in the pMN domain. These Olig genes are expressed in motor neuron precursors but not in differentiated motor neurons. Mice lacking both Olig1 and Olig2 function have no motor neurons or PDGFRα expressing oligodendrocyte lineage cells demonstrating that these Olig genes are required for the specification of both motor neurons and OLPs suggesting that motor neurons and oligodendrocyte share a common precursor cell (Richardson et al., 2000; Rowitch et al., 2002). Recently, Park et al (2002) showed that *olig2* is also required for the production of primary motor neurons and oligodendrocytes in zebrafish.

1.4.1.2 Oligodendrocyte origins: p3-pMN domain controversy in ventral spinal cord

Despite the increasingly convincing data for the ventral origin of most spinal cord oligodendrocytes, the precise ventral location of oligodendrogenesis (pMN or p3) is still debated. Some studies of chick spinal cord have suggested that oligodendrocytes might be generated from the Nkx2.2 domain of the ventral neuroepithelium, ventral to the PDGFRα/Olig1/Olig2/Sox10 domain and immediately adjacent to the floor plate (Fu et al., 2002; Qi et al., 2001; Xu et al., 2000). Evidence of expression of oligodendrocyte markers such as myelin proteolipid protein (PLP/DM-20) and monoclonal antibody O4, within the Nkx2.2 expression domain has also been reported in avians (Ono et al., 1995; Spassky et al., 2000). In chicken embryos it appears that Nkx2.2 expression expands from the p3 domain into the Olig2 domain, creating an area of overlap, which is where PDGFRα OLPS originate. In the mouse, however expansion of Nkx2.2 occurs after the first PDGFRα + OLPs have already left the VZ. Thus Nkx2.2 seems not to be required for OLP specification in mice (though it might be in chicks). This fits with the phenotype of Nkx2.2 null mice in which OLPs form apparently normally but fail to mature into oligodendrocyte suggesting that Nkx2.2 is required for oligodendrocyte differentiation. It remains possible however that there

might be different Nkx2.2 positive and Nkx2.2 OLPs with different properties (Fu et al., 2002; Qi et al., 2001; Soula et al., 2001; Zhou et al., 2001; Richardson et al., 2000; Spassky et al., 2000).

1.4.1.3 Oligodendrocyte origins: dorsal spinal cord

There is very recent evidence that the origins of oligodendrocytes in the spinal cord are from dorsal as well as ventral regions (Cai et al., 2005; Fogarty et al., 2005; Richardson, 2005). Cai et al, (2005) described oligodendrocyte production in Nk6.1 null mice. In spinal cord, Nk6.1 transcription factor was found to activate the bHLH transcription factor Olig2 in the pMN, which as previously noted (section 1.4.1.1) is necessary for the production of both motor neurons and OLPs. Nk6.1 null mice lose their ventral expression of Olig2 and lack ventral production of motor neurons and oligodendrocytes. However, PDGFRα and Olig2 cells continue to be produced in the dorsal spinal cord of these Nk6.1 null mice. The co-expression of Pax7 with Olig2 and PDGFRα positive cells confirms the dorsal oligodendrocyte origin. A second source of evidence for oligodendrocyte production in the dorsal spinal cord comes from green fluorescent protein (GFP) and Cre/LoxP fate mapping in transgenic mice. Fogarty et al (2005), using Dbx1-Cre X ROSA26-GFP mice demonstrated that Dbx1-positive precursors (in dP5/dP6 and v1, v0) generate a small number of oligodendrocytes about 3% of the total. Together the three studies (Cai et al., 2005; Vallstedt et al., 2005; Fogarty et al., 2005) suggest that up to ~10% of oligodendrocytes in the cord could be generated outside pMN. Therefore pMN produces ~90% of all spinal cord oligodendrocytes so it is still the major source (Richardson, 2006)

1.4.1.4 Specification, migration and differentiation of oligodendrocytes

After OLPs are produced from the neuroepithelium (during embryonic and early postnatal stages) they proliferate and migrate from the neuroepithelium into the grey and white matter of the spinal cord where they differentiate through a series of antigenically and morphologically distinct phenotypic stages culminating in myelinating oligodendrocytes (Pfeiffer et al., 1993). This differentiation follows a stepwise morphological transformation from bipolar progenitors to immature oligodendrocytes

bearing multiple processes, membrane sheath-bearing mature oligodendrocytes and, finally, myelinating oligodendrocytes. They are distributed without preference for dorsal, ventral, anterior or posterior position. Accompanying this oligodendrocyte differentiation and maturation is the sequential expression of molecular markers (See Figure 1.3). The A2B5 antibody (Eisenbarth et al., 1979) and NG2 (Nishiyama et al., 1999) is the first marker to appear in rodent, PDGFRα is then detected (Hall et al., 1996; Pringle and Richardson, 1993). As maturation proceeds OLPs begin to express O4 antigen which label mitotic as well as differentiated post mitotic oligodendroglia (Warrington and Pfeiffer, 1992; Warrington et al., 1992; Hardy and Friedrich, Jr., 1996a). Finally terminal differentiation is defined by the onset of galactocerebroside (GalC) (Ono et al., 2001) and 2',3'-cyclic-nucleotide 3'-phosphodiesterase (CNP) (Yu et al., 1994; Ono et al., 2001). Oligodendrocytes contact and then enwrap neighbouring axons, mainly in the white matter of the spinal cord, forming myelin sheaths around them and expressing high levels of myelin gene products. The most abundant proteins found in the myelin sheath in mammals are myelin basic protein (MBP) and proteolipid protein (PLP) in the CNS (Hartman et al., 1982). Slowly dividing undifferentiated OLP cells also persist in the adult spinal cord (Reynolds and Hardy, 1997). Figure 1.3 summarises those morphological and antigenic markers for oligodendrocytes during development.

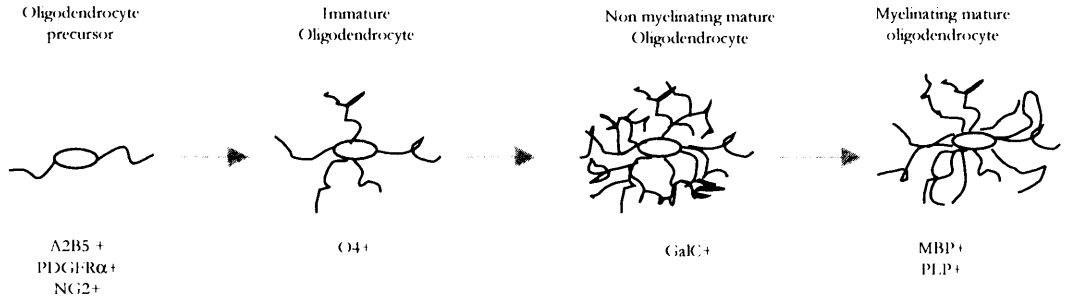


Figure 1.3 Schematic representation of the developmental stages of cells as oligodendrocyte. Schematic describing the main stages in oligodendrcotye development and sequential expression of some stage specific antigens. A2B5 antigen, platelet derived growth factor receptor-α (PDGFRα) and NG2 in precursors, O4 antigen in immature oligodendrocytes, galactocerebroside (GalC) in non-myelinating mature oligodendrocyte and myelin basic protein (MBP) and proteolipid protein (PLP) in myelinating mature oligodendrocytes. Adapted from Miller, 2002.

1.4.1.5 Oligodendrocyte origins in the forebrain

Oligodendrocytes are found everywhere in the CNS including higher structures of the brain such as the neocortex. Their origin, however, is less clear than in the well researched spinal cord. A number of similarities are found between the brain and spinal cord. For instance, in both cases oligodendrocytes appear to arise from ventral sources, as suggested by *in vitro* cultures in rats where only ventral telencephalic regions were able to give rise to oligodendrocytes (Tekki-Kessaris et al., 2001). Also, oligodendrocyte lineage markers such as Olig1, Olig2, Sox10 and PDGFRα are found expressed in cells in the ventral VZ of the basal forebrain. These labelled cells then spread laterally and dorsally throughout the forebrain including the cerebral cortex (Tekki-Kessaris et al., 2001; Woodruff et al., 2001). The similarity to the spinal cord continues with Shh signalling apparently required for oligodendrocyte development in both spinal cord and forebrain (Tekki-Kessaris et al., 2001; Pringle et al., 1996; Nery et al., 2001; Orentas et al., 1999). A common lineage with neurons has also been proposed for oligodendrocytes and GABAergic neurons (He et al., 2001) analogous to the oligodendrocyte motor neuron lineage proposed in the spinal cord (Lu et al., 2002; Richardson et al., 2000; Rowitch et al., 2002; Zhou et al., 2000; Richardson et al., 1997) (see section 1.4.1).

As with the controversy about the origin of oligodendrocytes in the spinal cord, similar debate is going on about the brain. It was suggested that besides ventral origins, oligodendrocyte production may occur in other brain regions (Olivier et al., 2001; Spassky et al., 1998; Spassky et al., 2000; Spassky et al., 2001). Only recently Cre-lox mapping studies using Emx1-Cre transgene mice suggest cortical oligodendrocytes arise from endogenous cortical precursors (Kessaris et al., 2005; Gorski et al., 2002). Kessaris et al, 2005 suggest that in fact, the cortex has several waves (ventral and dorsal) of oligodendrocyte production. A first wave before birth (by E18) is derived from ventral precursors in the medial ganglionic eminence (MGE), a second wave of OLPs originating in the cortex from the lateral ganglionic eminence (LGE) and third a wave from the within the cortex itself. By adult stages they describe that the ventrally derived population is eliminated from the cortex. A cortical production of oligodendrocytes was previously suggested by other studies which identified precursors cells in the subventricular zone of the brain lateral ventricles after birth (Levison et al., 1993; Parnavelas, 1999; Ivanova et al.,

2003). Figure 1.4 shows a representation comparing the development of OLPs in the mouse spinal cord and telencephalon.

This second wave of cortical precursors seems to be present only in rodents since chicken-quail grafting suggests that in birds all oligodendrocytes in the cortex develop from ventrally derived precursors (Olivier et al., 2001). It is probable that mammalians, with their expanded telencephalon need a larger number of oligodendrocytes and this provided selection for evolution of additional focal sources of precursors (Richardson, 2006).

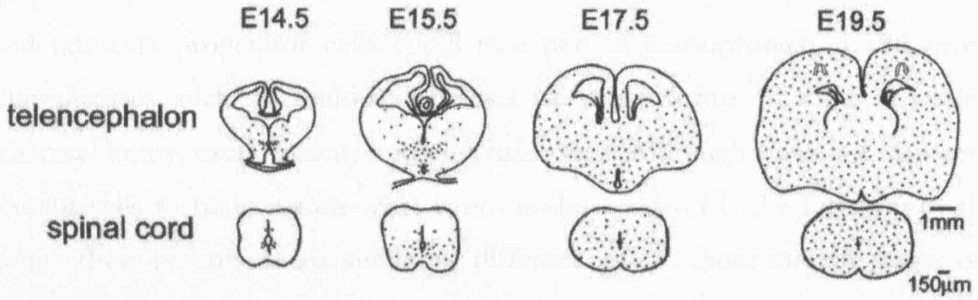


Figure 1.4 Schematic comparison of the development of oligodendrocyte progenitors in the mouse spinal cord and telencephalon. In both telencephalon and spinal cord, PDGFRα OLP cells (indicated in grey dots) have a ventral origin and spread into the surrounding mantle zones via proliferation and migration. Red dots represent a small percentage of OLPs that appear to originate in the dorsal part of the spinal cord. Red arrows represent the second wave of origin of oligodendrocytes in the cortex after birth. Adapted from Woodruff et al., 2001.

1.4.1.6 Oligodendrocyte progenitor cells in the adult brain

Oligodendrocyte progenitor cells have not only been found in the developing CNS, there is also a small population of slowly proliferating precursors that persists throughout the adult CNS (Levine et al., 2001). A number of oligodendrocyte molecular markers identify these precursors in the adult CNS: NG2 and PDGFRα are two antigenes shared by oligodendrocyte precursors that are found in the mature mammalian spinal cord and brain

CNS, suggesting the immature state of these cells (Nishiyama et al., 1999; Nishiyama et al., 2002; Watanabe et al., 2002). Progenitors have also been found in the subventricular zone of the brain lateral ventricles (Levison and Goldman, 1993) as well as dispersed throughout the subcortical and cortical parenchyma (Levine et al., 2001; Gensert and Goldman, 1996; Reynolds and Hardy, 1997) and in the optic nerve (Vaughn and Peters 1968). It has been shown that cells expressing the antigen NG2 undergo proliferation in demyelinated diseases (Watanabe et al., 2002). Nkx2.2, a homeodomain transcription factor, whose co-expression with Olig2 positive cells has been shown to be required for the maturation of OLP, is also found abundantly expressed in a subpopulation of OLPs in the adult rat spinal cord (Watanabe et al., 2004). Nkx2.2 appears transiently up-regulated in OLP cells as they differentiate into mature oligodendrocytes in the spinal cord white matter during remyelination (Watanabe et al., 2004) probably providing new oligodendrocytes to replace those that have been lost. It seems possible that these adult oligodendrocyte progenitor cells could take part in neuroprotection and myelin repair during diseases such as multiple sclerosis or other forms of CNS damage such as mechanical injury, excitotoxicity and viral infections. Although there is a response of adult precursor cells to brain insults what is not understood yet is the function of these cells. Whether these precursors are similar or different cells to those in early stages or whether they have the same or different characteristics and functions in the adult CNS remains unclear.

Recently, Arnett et al, (2004) have shown a possible role of Olig1 in remyelination of the adult CNS. They found that antibodies recognising Olig1 and Olig2 proteins localised in the nuclei of cells at all stages, but nearly all the Olig1 protein moves from the nucleus to the cytoplasm from two weeks after birth, when OLPs differentiate into oligodendrocytes and produce myelin. In addition, they found nuclear Olig1 protein at the edges of experimentally demyelinated regions in mice and in post mortem tissue from multiple sclerosis patients. These results suggests that the presence of Olig1 in the nuclei of cells might be needed for repair of the damaged CNS, as Olig1 protein was subsequently found in the cytoplasm in repaired tissue (Arnett et al., 2004).

1.4.1.7 Myelination by oligodendrocytes

A feature of oligodendrocyte differentiation is myelinogenesis, occurring perinatally in human and mice and continuing throughout early life. Myelination emerges in the white matter tracts in a regionally specific manner, maturing in a rostro-caudal pattern of development (Schwab and Schnell, 1989).

Electron microscopy (EM) and inmunohistochemical studies show that myelin formation starts with a net of thin processes surrounding selected axons. As myelination proceeds, a subset of the processes thicken and compact by the tight apposition of the membrane surfaces forming alternating major dense lines (MDL) and intraperiod lines (IPL), characteristic of the mature myelin (Xu et al., 2000; Hardy and Friedrich, 1996).

To reach this tight apposition (structure), a strong adhesion between the membranes is required, this is provided partly by proteins that are basic to the myelin membrane or associated with it. One of the key components is PLP, a "four-helix span" (Klugmann et al., 1997) membrane protein that forms 50% of the protein in CNS myelin. PLP and its shorter splice variant DM-20 are required for stabilizing the apposition of the adjacent extracellular membrane surfaces by forming the double-spaced at the IPL (Gow et al., 1997; Griffiths et al., 1998; Yool et al., 2001). MBP is the second most abundant protein in the mammalian CNS. It is also detected in the PNS, where it represents a small percentage (10-20%) of proteins present (Baumann and Pham-Dinh, 2001). MBP is localized in the MDL playing a crucial role in the compaction of the surfaces of the plasma membrane processes at this site, in the CNS (Omlin et al., 1982). Whereas in PNS myelin this task is performed by a major integral membrane glycoprotein termed myelin protein zero (P_0), an Ig-like adhesion molecule (D'Urso et al., 1990; Lemke and Axel, 1985; Lemke, 1988).

1.4.2 Astroglia cells

Astrocytes (the most abundant cells within the CNS) are the delicate multiprocessed glial cells found throughout the CNS. These were first visualized by Adriezen (1893) and noted by their complex shapes in which thick processes branch into thinner ones. Although the

origins and general development of oligodendrocytes are beginning to be illuminated, the studies of astrocyte spatial regulation and lineage are still in their infancy.

It was long thought that astrocytes were passive cells, being important for supporting the cytoarchitecture of the brain (Montgomery, 1994). This view is gradually changing to astrocytes as a heterogeneous population that have roles in numerous physiological and structural functions in neural development. Astrocytes provide metabolic support, being important for the uptake of K+. Furthermore, they can respond to neurotransmitter release e.g. glutamate. They maintain the extracellular ionic environment for neuronal function, balance the pH, maintain blood brain barrier, recycle neurotransmitters, store energy and regulate vasculature. They are also responsible for a multitude of trophic functions (Powell and Geller, 1999; Powell et al., 1999; Vernadakis, 1996). More recent findings indicate that astrocytes are also necessary for synapse formation and synaptic modulation (review in Ramson et al 2003; Newman, 2003; Fields and Stevens-Graham, 2002).

Generally astrocytes are identified by their stereotypical stellate shape and characteristic intracellular expression of the intermediate filament glial fibrillary acidic protein (GFAP) at around E16 (in mice; (Bignami and Dahl, 1974).

Astrocytes are classically divided into protoplasmic astrocytes found in grey matter and fibrous astrocytes in the white matter. They can then be further divided into several other subtypes (Wilkin et al., 1990) with at least five distinct types of astrocytes identified from rat spinal cord culture (Miller and Szigeti, 1991). How do all these types of astrocytes arise? Are there one or multiple classes of precursors?

1.4.2.1 Astrocyte developmental origins

It is known that neurons and glial cells arise from the neuroepithelium of neural tube. In early development, radial glial and neurons are the first to appear, followed by OLPs and astrocytes. Similar to oligodendrocytes, astrocytes are also found distributed throughout the grey and white matter of CNS and are diverse in form and function. Astrocytes continue to be generated throughout the lifespan of the animal.

A review of the literature yields proposals for two different sources of astrocytes: (1) from the radial glia that arise around the time of neurogenesis and participate in neuronal migration via transdifferentiation after their initial role is completed (Goldman et al., 1997; Voigt, 1989; Gaiano et al., 2000) and (2) from immature cells in the subventricular zone (Pringle et al., 2003).

A number of shared characteristics between radial glia and astrocytes suggest that these two cells are related. Among these characteristics is the similar expression pattern of molecular markers such as nestin, vimentin and GFAP (in primates) found in both types of cells (Levitt et al., 1981; LeVine and Goldman, 1988). Both express glutamate transporters (Shibata et al., 1997; Yamada et al., 1998). Further, the appearance of astrocytes in white matter coincides with the disappearance of radial glia (Choi and Lapham, 1978; Misson et al., 1988; Misson et al., 1991) suggesting a precursor-product relationship. Different groups have been able to follow transformation of radial glia into astrocytes (Voigt, 1989; Gaiano et al., 2000).

The lack of astroglial precursors markers makes it difficult to directly discern the spatial origin of these cells. Recently, Pringle et al, (2003) proposed Fibroblast Growth Factor Receptor 3 (FGFR3) as a marker for astrocytes and their precursors in both chick and mouse embryonic spinal cord (Pringle et al., 2003). FGFR3 expression localised astrocyte precursors in the ventral and dorsal VZ of the embryonic spinal cord except in the pMN domain that gives rise to motor neurons and oligodendrocytes. Then, FGFR3 positive cells distribute into both grey and white matter. FGFR3 labelled cells were also double labelled with GFAP positive astrocytes *in vivo* and *in vitro*, but never with PDGFRα positive oligodendrocyte progenitors suggesting specificity of FGFR3 for astrocytes only. Unlike oligodendrocytes Shh appears to be unnecessary for the production of spinal cord astrocytes. However, like oligodendrocytes they retain the ability to proliferate after leaving the VZ (Pringle et al., 2003). They also showed that FGFR3 positive cells within and outside the VZ also express the astroglial marker glutamine synthetase (GS). This dorsal and ventral neuroepithelial astrocyte origin is in accordance with previous results (Pringle et al., 1998) describing ventral and dorsal parts of the neuroepithelium giving rise

to astrocytes in chick-quail, chimeras, whilst oligodendrocytes arise only from the ventral regions.

1.4.2.2 Astrocyte developmental origins; neuron-astrocyte lineage

As with oligodendrocytes the origins of astrocytes have also been linked to neuronal lineage. In studies of mice null for bHLH transcription factors Olig1 and Olig2, the pMN domain is converted into the adjacent domain p2 generating interneurons and then astrocytes (Zhou and Anderson, 2002; Takebayashi et al., 2002). Oligodendrocytes may be respecified as astrocytes in the absence of Olig1 and Olig2 genes revealing evidence for a neuron-astrocyte lineage. Cells in the pMN domain, Olig genes, repress the astroglial fate during development, so astroglia specification can only occur in the absence of these inhibitors. Similar to oligodendrocytes, astrocyte specification is the subject of inhibition by neurogenic factors (Sun et al., 2000).

1.4.2.3 Astrocyte developmental origins; glial restricted precursors.

Rao and colleges (1998) have maintained that neither oligodendrocyte nor astrocyte cell origins are linked to neurons. Instead they proposed that both oligodendrocytes and astrocytes arise from multipotent neuroepithelial cells via intermediary precursor cells, termed glia restricted precursor cells (GRPs) (Rao et al., 1998). They isolated GRP cell population from spinal cords of E13.5 rats. *In vitro*, these A2B5 GRP cells can undergo extensive proliferation and then generate two distinct types of astrocytes and oligodendrocytes but do not differentiate into neurons. Since their experiments were mostly *in vitro*, it is difficult to explain their different results from those experiments done *in vivo*. It is possible the cells behave differently when they are transfered to *in vitro* system. What is clear is that the data from this study are in contrast with the results from Olig1/Olig2 knock outs (Zhou and Anderson, 2002; Lu et al., 2002). These knockouts produced revealed absence of motor neurons and oligodendrocytes from the pMN domain, but astrocyte (by GFAP staining) appeared normal. However there is a lack of specific markers for the early stages of oligodendrocyte development making these studies difficult to interpret.

1.4.2.4 Astrocyte specification

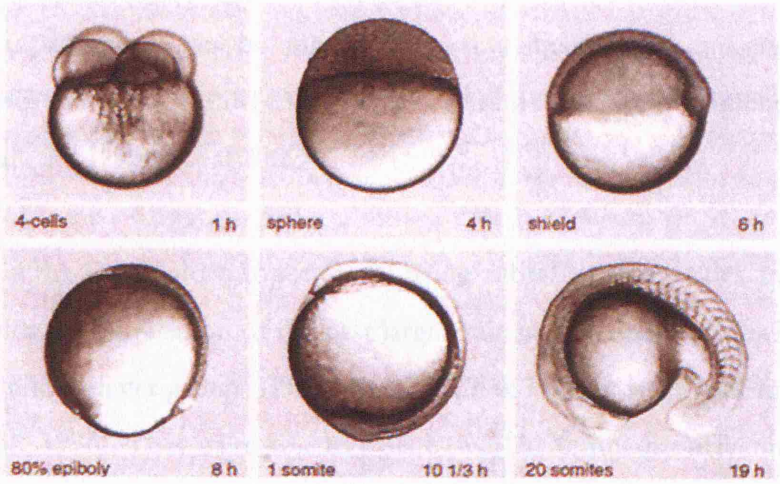
The identification of early or mature astrocytes *in vivo* still remains problematic; there are very few markers available. GFAP is still the most widely used, although the use of GFAP immunoreactivity as a defining characteristic of *in vivo* astrocytes is inconsistent. There are at least three other populations of cells that exist in the adult brain that are reported to express GFAP: ependymal, adult neural stem cells, and adult radial glia cells (Walz and Lang, 1998; Alvarez-Buylla and Lois, 1995; Doetsch et al., 1999). GFAP is also expressed in embryonic radial glial cells in other species (Messenger and Warner, 1989). Additionally, some astrocytes are not GFAP positive (Walz and Lang, 1998; Stichel et al., 1991).

1.5 Zebrafish (*Danio rerio*), a vertebrate model organism for developmental genetics.

Zebrafish, *Danio rerio*, is a freshwater tropical teleost fish originally found in slow streams and rice paddies in the Ganges River, East India. In the past decade or so the zebrafish has become a widely used model system for vertebrate development and genetics and is often referred to as the new *Drosophila* of vertebrate developmental biology (Eisen and Weston, 1993; Eisen, 1996; Driever et al., 1994). The zebrafish has been chosen as a model for the studies of this Thesis because of their many advantages, as described below:

1. External fertilization and development makes almost all developmental processes easily accessible.
2. The translucent embryo allows monitoring of morphological changes directly in living or fixed embryos. For example, whole mount *in situ* hybridization can be used to examine three-dimensional patterns of tissues showing specific gene expression.
3. They are easy to raise and maintain at high density.
4. Zebrafish development is fast, taking only three days from fertilization to hatching.
5. They are sexually mature within three months and a single pair of adults can produce 100-200 offspring per week.

6. Cells can be labelled in living embryos, so patterns of division and migration as phenotypes can be observed and characterized.



7. Single or groups of cells can be transplanted to new locations at appropriate developmental stages where intrinsic cellular and extrinsic environmental factors in development can be assessed.

8. Zebrafish are remarkably permeable to small molecules added to water, an advantage in the study of the interactions of genes and environment and pre-clinical drug discovery as well as toxicological evaluation. Zebrafish could be key to understanding adult human diseases such as heart failure, cranio facial disorders, pigmentation or eye defects (Penberthy et al., 2002).

9. The zebrafish genome will be completely sequenced within the next 1-2 years. www.sanger.ac.uk.

10. Possibility of making large-scale mutagenesis screens that affect various aspects of vertebrate embryonic

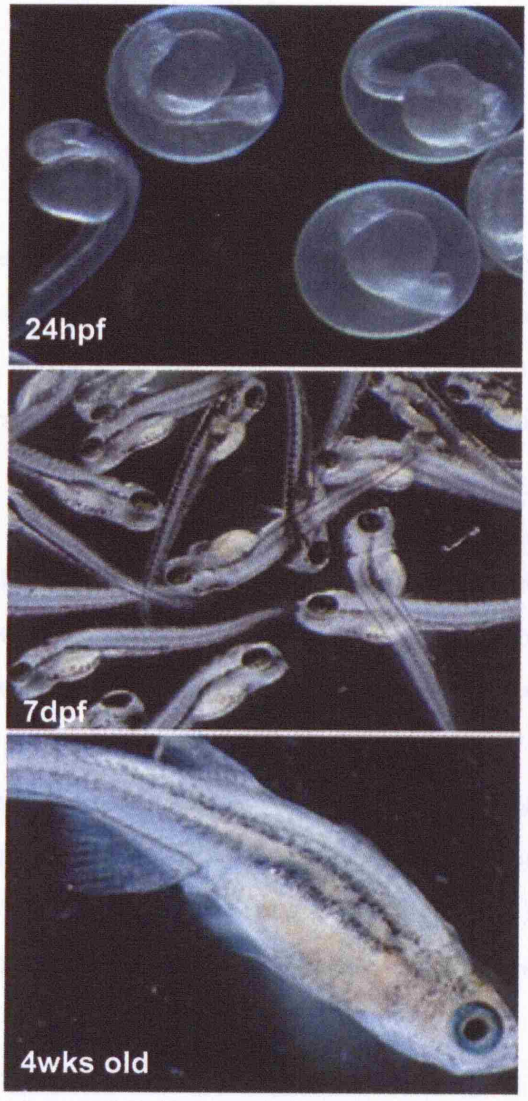


Figure 1.5 Zebrafish (*Danio rerio*) digital photographs at different stages of development.

development providing the ability to identify genes which regulate it.

11. Gene function in zebrafish can be inhibited by the injection of antisense morpholino oligonucleotides that block mRNA splicing or translation in a sequence specific manner (Lewis et al., 2001; Park et al 2002).

Streisinger et al., (1981) was the first major proponent of using zebrafish as a model, but interest began in earnest with the publication of the first large scale mutagenesis screens in 1996 by Nusslein-Volhard and Driever groups (Driever et al., 1996; Haffter and Nusslein-Volhard, 1996; Haffter et al., 1996). They were able to isolate over 2000 mutants affecting a large variety of processes such as embryonic development, organ formation and simple behaviour.

1.6 Aims of this work

In addition to other suitable characteristics, described above, the embryonic transparency, high fecundity and the possibility of performing high mutant screens make zebrafish an attractive model for studying gene function and identifying new components of the genetic pathways regulating developmental processes such as glia. However, little is known about glia development in fish.

The primary goal of this study is to establish the baseline for zebrafish as model for glial development. I investigated the developmental origin and mature stages of oligodendrocytes in embryonic and adult zebrafish by studying in detail the temporal and spatial expression pattern of oligodendrocytes precursor markers (*olig1, olig2, nk2.2, sox10* and *pdgfrα*) and myelin genes involved in oligodendrocyte maturation (*mbp, plp* and p_0). The genes *olig1* and *olig2* were cloned for this purpose. I also performed double labelling experiments between some of these genes and spinal cord patterning genes (*iro3, pax6*). Furthermore, I studied *Sox10* and *Pdgfrα* in a similar manner in relation to oligodendrocytes development in *Xenopus laevis.*

The expression pattern of the oligodendrocyte lineage markers such as, *olig2*, *sox10*, *nk2.2* and *mbp* were examined in the spinal cord of mutant zebrafish *otter* (ott::) in which a myelin defect has been observed. Electron microscopy was also used to verify whether sheaths of myelin are present in these mutants in comparison to the wild type zebrafish.

One of the major differences between mammals and zebrafish is the lack of *pdgfrα* in the CNS of zebrafish (Liu et al., 2000). The differential expression patterns of Pdgfrα gene in mammals compared to zebrafish was investigated, invoking a transgenic approach to studying regulatory elements of these genes by injecting at the single cell stage in zebrafish. I purified, injected and analysed the transient mosaic expression in zebrafish of a human bacterial artificial chromosome (BAC) DNA construct containing the PDGFRα gene, which drives OLP specific expression in mouse.

Secondary to the study of oligodendrocytes, I investigated the origin and mature stages of astrocytes in the developing and adult zebrafish. The astrocytic markers used herein are *gfap*, *gs* and *fgfr3* to identify their expression patterns from post fertilization until one month of age. *Xenopus laevis*, astrocyte development was also analysed using these gene homologues.

Chapter 2

Experimental Procedures

Unless otherwise listed, all chemicals used were obtained from Sigma-Aldrich Company Ltd, Poole, UK.

2.1 Molecular biology

2.1.1 Bacterial strains, growth and storage

Cloning was carried out using Escherichia coli *(E.coli)* strain XL1-Blue *(recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac [F' probAB, lacI^{q}ZΔM15, Tn 10 (Tet^{R})])*. Bacteria grew at 37°C in Luria Broth (LB; per litre: 10g bactor-tryptone, 5g bacto-yeast extract and 10g NaCl) or on LB-agar plates containing LB with 15g/l bacto-agar. After cooling to 55°C Ampicillim was added to the LB at a final concentration of 25-100ug/ml (100mg/ml stock in H_2O, 0.20μm filter-sterilised and stored in aliquots at –20°C). Liquid cultures were continually agitated in a rotating environmental shaker (Innova TM 4330 from New Brunswick Scientific) at 3000 rpm. Short term storage of bacterial strains and clones were on LB agar plates at 4°C for two or three weeks. For long-term storage, glycerol was added to overnight cultures to a final concentration of 15%. This mixture was stored in 1ml aliquots at –80°C for extended periods including, if necessary, for a several years.

2.1.2 Agarose gel electrophoresis of DNA

Nucleic acid size determination and/or separation were performed by agarose gel eletrophoresis. Agarose gels were prepared by dissolving multi-purpose agarose (Bioline) in 1x TAE (50X TAE: 2M Tris-acetate pH7.5, 50mM EDTA) buffer to a final concentration 0.8-1.5% (w/v) depending on the expected size of the DNA fragments. The agarose was boiled in a microwave oven and then cooled to below 60°C before adding ethidium bromide at a final concentration of 0.5μg /ml. The agarose was then poured into a horizontal electrophoresis system (Life Technologies Ltd) and left to set in the cold room at 5°C. 10x DNA loading buffer containing 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF and 15% (w/v) Ficoll (Type 400, Pharmacia) was added to the DNA samples. Once loaded, the gel was run at 140V. Electrophoresis was performed at 5-20V/cm gel length, until the appropriate resolution was achieved. Ethidium bromide-stained nucleic acid was visualised using ultraviolet

light (λ= 302nm) and fragment size was estimated by comparison with 1kb ladder molecular weight markers (Gibco BRL) run in at least one of the gel lanes. A gel documentation system (Alpha Innotech Corporation) with an ultra violet transilluminator at 302nm and CCD camara was used to visualize the DNA.

2.1.3 Small scale preparation of plasmid DNA by alkaline lysis (miniprep)

Bacterial colonies grown on a LB-agar plate were picked using sterile Gilbson pipette tips, which were then ejected into sterile 25ml Universal tubes containing 5ml LB with ampicillin. Cultures were grown at 3000rpm overnight in a 37°C shaking incubator. Then, 1ml of culture was transferred to an Eppendorf tube and centrifuged at 13,000rpm in a microcentrifuge (Eppendorf 5415D) for 2 minutes. After removal of the supernatant, the bacterial pellet was resuspended in 100μl of ice-cold "solution I" (50mM glucose, 25mM tris-HCl pH8, 10mM EDTA) and incubated on ice for five minutes. Then 200μl of freshly prepared "solution II" (1% Sodium Dodecyl Sulphate (SDS), 0.2N Sodium Hydroxide (NaOH)) was added and mixed by several gentle inversions of the tube. The mixture was kept on ice for a further five minutes. 150μl of "solution III" (3M potassium acetate and 2M acetic acid) was then added, gently mixed by shaking, and the tube again incubated on ice for five minutes. After spinning for 5min at 13000rpm, the supernatant was carefully removed, avoiding the floating material, and transferred to separate tube. The plasmid DNA was precipitated with 2x volume ethanol at –20°C for 10 min. This was spun down at high speed (13000rpm) for 10 min and the pellet washed out with 70% ethanol and centrifuged again. The DNA was then air dried and resuspended in 40-50μl TE buffer pH 7.6, from which 2-5μl could be used per 20-30μl restriction enzyme digestion to check the plasmid DNA. Restriction enzyme digestion of DNA was carried out as per the enzyme manufactures' protocol (New England Biolabs Ltd or Promega Ltd) in reaction buffers supplied by the manufactures.

2.1.4 Large-scale preparation of plasmid DNA (midiprep and maxipreps)

This method was a scaled-up version of the small-scale plasmid isolation method described above with several additional plasmid purification steps. The remainder of the mini growing bacteria was diluted in 100 ml (midiprep) or 250 ml (maxiprep) of LB with ampicillim in a one litre flask and grown at 37°C in a rotatory shaker overnight. A plasmid purification kit was used, based on a modified alkaline lysis procedure, followed by DNA binding to an anion-exchange resin (Qiagen Ltd). The procedure was carried out as described in the manufacture's handbook.

2.1.5 Quantitation of genomic DNA

To calculate the concentration and purity of the plasmid DNA, the optical density of a sample was measured using a spectrophotometer (Shimadzu). After diluting 1:200 in water, the optical density at 260nm and 280nm (OD^{260}, OD^{280}) was taken and concentration calculated as follows: [OD^{260} x 200 (dilution factor) x 50 (DNA multiplication factor)] µg/ml. To assess the purity of each sample, the ratio OD^{260}/OD^{280} was calculated and found to be between 1.7 - 1.8 for pure DNA.

2.1.5.1 Gel extraction of DNA

For the extraction of DNA from agarose gels the Qiacuick Gel extraction Kit (Quiagen) was used according to the manufacture's protocol.

2.1.6 Isolation of genomic DNA

7dpf fish were transfer into a microfuge tube and rinsed with H_2O. As much liquid as possible was removed and 500 µl extraction buffers added. They were then incubated at 50°C minimum 3 hours mixing occasionally. DNA preparation buffer (10 mM Tris pH 8.2 10mM EDTA, 200mM NaCl, 0.5% SDS, 200 µg/ml proteinase K) with 1ml ethanol was added and placed on ice for 20-30 minutes. After this time, samples were centrifuged at 13000rpm for 10 minutes at 4°C. Pellets were washed in 70% ethanol by spinning for another 2 minutes, diluted in 1ml of TE and stored at 5°C. The concentration was then determined.

2.2 Polymerase chain reaction (PCR)

PCR was performed in a Master Cycler Gradient from Eppendorf. For a 25µl amplification reaction the reagents were as follows: 2.5µl 10xPCR buffer (500mM KCl, 100mM Tris-HCl pH9. 0.1% (v/v) Triton X-100; Promega), 0.1µl of *Taq* DNA polymerase (5U/µl; Promega), 0.25µl dNTPs (20mM each of dATP, dCTP, dGTP, dTTP; Amersham Pharmacia Biotech), 1.5µl of 25mM Magnesium chloride (Promega), 0.1µl of each primer (100pmol/µl; MWG Biotech) and a suitable concentration of DNA template were mixed. The PCR amplification conditions were 35 cycles performed with the melting temperature at 94°C for 45 seconds, annealing temperature at 56°C for 45 seconds, elongation at 72°C for 1 minute and then 72°C for 10 minutes. Positive and negative controls (i.e. complete reaction mixtures lacking template DNA, with H_2O) were always set up simultaneously. The resulting reactions were run on a 1% agarose gel in 1xTAE buffer stained with ethidium bromide to check the PCR products.

olig2	Zo1	ACG AGT GAA CTG GAT AGC CT
	Zo2	GCT TCA TCT CCT CCA GCG AG
	Zo4	CCG GAG CCG GTC CAT GCC GA
	Zo5	TCC TCC ACG CAG AGC GAT TC
	Zo6	AAA CTG AGA GCG CAC TGA AC
sox10	F	ACC GTG ACA CAC TCT ACC AAG ATG ACC
sox10	R	CAT GAT AAA ATT TGC ACC CTG AAA AGG
olig1	F	TCA GAA TGC AGG CTG TGT CTG GTG
olig1	R	TCG GAA AAC GCA TGG CTG GAT

Table 2.1: PCR primers table.

2.2.1 DNA sequencing

DNA sequencing was carrying out by automated sequencing. The DNA sequencing service was provided by the Wolfson Institute for Biomedical Research, www.wibr.ac.uk and was performed by a Beckman Coulter CEQ2000XL Sequencer.

2.3 Cloning techniques

2.3.1 Isolation of DNA fragments for cloning

DNA fragments were purified from agarose gel in which they had been run after PCR amplification. Bands were visualised on the agarose gel using UV light and with a clean scalpel, the slice of agarose containing the DNA band was excised to then be purified. A kit from Amersham Biosciences was used. Instructions from the commercial protocol were followed.

2.3.2 Preparation and transformation of competent bacteria

The ligation reactions were performed using TOPO TA Cloning (Invitrogen Life Technologies) and according to the manufacturer's instructions.

The cloning reaction was performed according to the following conditions: 4µl fresh PCR product or 4µl of gel purified product, 1µl of 1M NaCl solution and 0.5 pCR at TOPO vector. These were mixed gently and incubated for 5 min or overnight at room temperature.

2µl of the TOPO Cloning reaction was used to transform into One Shot E. coli. The mix was incubated on ice for 5-30 minutes and then the cells were heat-shocked for 30 seconds at 42°C without shaking. Then, 250µl SOC were added and these incubated at 37°C for 1 hour in a rotating shaker before plating on LB-agar-ampicillin plates.

Blue/white selection was used to distinguish recombinant from non-recombinant plasmids. Each plate was spread with 40µl of 1.25% (w/v) X-gal (5-bromo-4-chloro-3-indolyl-Beta-D-galactopyranoside) and 10mM IPTG (Isopropyl Beta-

thiogalactopyranoside) and incubated at 37°C for 30 minutes before use. The X-gal stock was 2.5% (w/v) in dimethyl formamide and was kept at –20°C in the dark. The IPTG stock was 0.1M in H_2O and was stored at 4°C. Colonies containing vectors were white under these conditions.

2.4 *In situ* RNA hybridization

This technique was used to analyse mRNA expression in cells and therefore it was essential to ensure that all solutions and equipment used were RNase free to protect the mRNA as much as possible. Solutions were treated with diethylpyrocarbonate (DEPC) made up to 0.1% (v/v) and then autoclaved. Those solutions that could not be autoclaved were made up in DEPC treated water. All dissection equipment was washed in DEPC treated water and all plastic ware used was sterile and kept sealed until just prior to use.

2.4.1 Preparation of digoxygenin-labelled probes

Dioxigenin-labelled RNA probes were prepared by *in vitro* transcription from the linearized plasmids with the inclusion of dioxigenin-UTP using a DIG RNA labelling kit (Boehringer Mannheim).

Species	Probe	Restriction enzyme used	RNA polymerase used
Zebrafish	*shh*	HindIII	T7
Zebrafish	*iro3*	Sal 1	T7
Zebrafish	*pax6*	Sma1	T7
Zebrafish	*sox10*	BamH1	T7
Zebrafish	*olig2*	Xho1	T7
Zebrafish	*olig1*	BamH1	T7
Zebrafish	*nk2.2*	BamH1	T7

Zebrafish	*pdgfrα*	Not1	T3
Zebrafish	*pdgf-a*	SacII	SP6
Zebrafish	*fgfr3*	Xho1	SP6
Zebrafish	*mbp*	Sal1	SP6
Zebrafish	*plp*	Sal1	SP6
Zebrafish	p_0	Sal1	SP6
Xenopus	*Sox10*	EcoR1	T7
Xenopus	*Pdgfrα*	Sma1	T3
Xenopus	*Fgfr3*	Not1	T7
Mouse	PDGFRα	Hind III	T7
Human	*PDGFRα*	EcorR1	T3

Table 2.2: DNA templates and enzymes used for probe production.

To first prepare the template, 10μg of cDNA (template DNA) containing the gene of interest was linearized with the suitable restriction enzyme for 2-3 hours. Linearization was checked by running the sample on an agarose gel. 5μl of 10% (w/v) SDS and 1μl of Proteinase K (20mg/ml) were added and the mixture incubated at 55°C for 15 minutes. Following this, the mixture (linearized DNA) was extracted twice with phenol: chloroform: isoamyl alcohol in the ratio 25:24:1. To do this, 100μl of phenol-chloroform was added to the digested template, mixed thoroughly and then centrifuged for 2 minutes at 13000 rpm. The aqueous phase was then isolated and extracted once with choloroform: isoamyl alcohol (24:1). To precipitate DNA, sodium acetate (final concentration of 0.3M) and twice the volume of ethanol were added. This mixture was kept at –20°C for 30 minutes and then centrifuged at 4°C for 10 minutes. The pellets were then washed in 70% ethanol, air-dried and dissolved in 50μl DEPC-treated dH_2O and stored at –20°C. *In vitro* transcription factors were set up at 37°C for 2 hours in the following order:

1. 1μg linearized template DNA
2. 5μl 5X Transcription Buffer (200mM Tris- HCl pH7.9, 30mM MgCl2, 10mM spermidine and 50mM NaCl) (Stratagene)

3. 7.5µl DTT (100mM stock)

4. 2.5µl DIG labelling mix (10mM of ATP, CTP, GTP, 6.5mM UTP, 3.5mM DIG-11-UTP; Boerhringer Mannheim and Roche)

5. 1µl RNAsin (Promega)

6. 2µl polymerase (T7, T3 or SP6 20 units as required; Promega)

7. Made up to 25µl total volume with distilled water.

To stop the reaction, 2µl of 200mM EDTA pH 8.0 was added and the reaction mixture made up to 100µl with DEPC treated water. One µl of the probe was run on an agarose gel in order to confirm full-length transcription. The remaining probe was stored at –70°C until use. The optimal dilution of each probe was determined by titration of the probe on a control section to achieve the maximum signal to noise ratio, usually between 1:1000 and 1:2000.

2.4.2 Embryo collection

Zebrafish (*Danio rerio*) female and male pairs were placed in tanks together in the evening preceding the desired collected day. They were obtained from the in-house zebrafish facility in the Department of Anatomy and Developmental Biology, University College London. They were maintained in 12 litre tanks with a water temperature of 28°C. The light cycle was 14hours light and 10 hours dark. The tanks were supplied with a constant feed of filtered water from a gravel bed and fluidised sand filtration plant and were constantly aerated. The fish were fed twice a day with a mixture of cultured brine shrimp and powdered flake fish food (Tetra-Min, UK). Larval zebrafish were obtained from the natural daily spawning of the breeding colony of the same facility. They were raised for the first 6 days in water containing 0.001% w/v methylene blue at 28°C. Following this they were transferred to the nursery where they were maintained in one litre tanks that were drip-fed with filtered water. They were fed cultured paramecia and finely powdered fish embryo food (ZM Ltd UK). The age of the larvae was counted from the day of fertilization. Eggs are usually laid and fertilised the following morning shortly after the lights were turned on. Embryos were collected in Embryo Water (red

sea salt 0.03g/ll, methylene blue (2mg/l) shortly after having been laid. Embryos were staged according to the morphological criteria provided in Kimmel et al. (1995). Zebrafish embryos that were collected for staining procedures were fixed at least overnight in 4% PFA phosphate buffer saline (PBS; 137mM NaCl, 2.7mM KCl, 4.3mM NaHPO4 7H20, 1.4M KHPO) at 4°C. Twenty-four hpf or older embryos were de-chorionated prior to fixation so their trunk and tail would be straight. Following fixation, embryos were dehydrated in increasing concentration of MeOH in PBS. Dehydrated embryos were stored in 100% methanol at -20C until used.

Preparation of tissue sections. Embryos were collected from pair matings, raised at 28.5°C and staged according to hours post fertilization and morphological criteria (Kimmel, 1995). To prepare sections, embryos following removal of chorions were placed in 4% paraformaldehyde in PBS and left at 4°C for 24 hours to fix. To cryoprotect, tissue was transferred to 20% sucrose in PBS and left at 4°C for a further 24 hours. Then, samples were embedded in OCT embedding compound (Sakura-Tissue-Tek) and frozen slowly on dry ice. Tissue blocks were stored at –70°C until required for sectioning. Frozen sections were cut 10-15μm thick. Cryosections on a cryostat (using a Microtome 5030; Bright Ltd) were collected on glass microscope slides, polysine (BDH) air-dried for 2 to 4 hours and then used for *in situ* hybridizations or immunohistochemistry.

Xenopus: Staging of tadpoles was determined according to Nieuwkoop amd Faber (1994). They were anaesthetized in 0.15% MS-222 diluted in Ringer's solution for 30-60 min, decapitated and the brain and spinal cord dissected.

2.4.3 *In situ* hybridization on tissue sections

Hybridization of sections with DIG-labelled probes. DIG-labelled antisense RNA probes were made as described before (Section 2.4.1). Probes were diluted in hybridization buffer usually at 1:1000, but a concentration series from 1:500 to 1:2000 was performed for each new transcription product. Hybridization buffer was made up as follows: 1x salts (10X salts: 2M NaCl, 50mM EDTA, 100mM Tris-HCl pH7.5, 50mM NaH_2PO_4, $2H_2O$, 50mM Na_2HPO_4), 50% deionised formamide (added 10% (w/v) 'Amberlite' IRN-150L monobed mixed resin (BDH) to formamide (Sigma) for 30

minutes, then filtered to remove beads), 0.1mg/ml yeast total RNA (Roche), 10% (w/v) dextran sulphate (Roche), 1x Denhardt's (50X stock, Sigma)

Once the probe was diluted, it was mixed and denatured for 5 to10 minutes at 75°C, then vortexed to ensure complete mixing. Approximately 250µl of probe solution was added to each microscope slide and a baked coverslip lowered on top avoiding the formation of air bubbles. Slides were kept in a sealed box on blotting Whatman paper soaked in 50% (v/v) deionised formamide/1XSSC and hybridised overnight at 60-65°C.

Post-hybridization washes and antibody staining: After hybridization, slides were transferred to coupling jars where they were incubated in wash buffer (1X SSC, 50% formamide, 0.1% Tween-20) pre-warmed to 65°C for at least 45 minutes. Coverslips were then removed easily and slides washed again in wash buffer at 65°C (twice for 30 minutes). Slides were incubated or washed twice in 1xMABT (5x stock: 500mM maleic acid pH7.5, 750mM NaCl, 0.5% (v/v) Tween-20) at room temperature (30 minutes each).

Slides were then transferred to a humidified chamber and incubated in blocking solution (MABT with 2% blocking reagent (Boehringer Mannheim) and 10% heat-inactivated sheep serum (Gibco BRL) for at least 1 hour at room temperature without a coverslip. The blocking solution was then replaced with alkaline phosphatase-conjugated anti-digoxygenin AP-conjugated antibody (Fab fragments; Roche Boeringher Mannheim), diluted 1:1500 in blocking solution and placed onto slides incubating overnight at 4°C.

Post antibody washes and colour reaction: To visualised hybrids, the slides were transferred into coplin jars again and washed 5 x 20 minutes in MABT at room temperature. A further two washes, 10 minutes each in prestaining buffer (100mM Tris-HCl pH9.5, 100mM NaCl, 50mM $MgCl_2$) made up fresh. Slides were then incubated in the dark in staining buffer (100mM Tris-HCl pH9.5, 100mM NaCl, 50mM $MgCl_2$, 5% polyvinyl alcohol (Sigma), 0.2mM 5-bromo-4-chloro-3-indolyl-phosphate (Roche), 0.2mM nitroblue tetrazolium salt (Roche)) until staining was satisfactory. To stop the

reaction, slides were rinsed in several changes of water for 30 minutes, and then mounted under coverslips in agar media (Agar Scientific).

2.4.4 *In situ* hybridization in wholemount zebrafish embryos

Embryos were collected as before, chorions removed and placed in PFA for 24 hours. Embryos were then stored at -20 °C in methanol. Wholemount embryos once removed from storage in –20°C methanol were washed twice in PBS/0.1% Tween-20 for 10 minutes. Embryos were then slightly digested from 5 to 12 minutes in proteinase K in PBS at room temperature, diluted 1:200 depending on the developmental stage of the fish (younger stages being more sensitive). The protocol followed was as previously described (Section 2.4.2), however whole embryos were transfered in small Eppendorf tubes during the procedure. Once the staining was satisfactory, completed embryos were washed into 70% glycerol/PBS and stored at -20°C.

2.4.5 Double *in situ* hybridization in sections

Double RNA *in situ* hybridization were performed using the combination of DIG and FITC RNA probes to examine the relationship and interaction between genes and their role in development of the spinal cord. Two different probes were labelled either with digoxigenin-11-UTP or FITC-12-UTP. The first probe was detected with alkaline phosphatase (AP) conjugated anti-digoxigenin antibody developed with Nitro Blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP), which yields a purple precipitate. The second probe was detected with anti FITC-AP conjugated antibody developed with p-Iodonitrotetrazolium violet (INT) and BCIP, which yields a brown/orange precipitate.

Once the first probe was developed (Section 2.4.2.) the AP enzyme was killed by heating the sections at 65°C in MABT for 30 minutes (or longer). Slides were washed twice in 1xMABT (as in Section 2.2.2) at room temperature (30 minutes each). They were then incubated for 1 hour in blocking solution at room temperature and overnight at 4°C in alkaline phosphatase-conjugated anti-digoxygenin AP-conjugated antibody (Fab fragments, Roche, Boeringher Mannheim) diluted 1:1500 in blocking solution.

Slides were transferred into coplin jars again and washed as described in Section 2.4.2, then incubated in INT/BCIP (Roche) in 37°C until developed. To stop the reaction, slides were rinsed in several changes of water for 30 minutes, and then mounted under coverslips in agar media (Agar Scientific)

2.5 Immunohistochemistry

Immunohistochemistry was used to label different type of cells in the zebrafish (neurons and astrocytes). Sections were treated at room temperature, permeabilised for 15 min in 1% triton-X-100 in PBS (PBS-T), then incubated for thirty minutes in 4% normal goat serum in 1% PBS-T. Sections were incubated overnight in primary antibody (see list below). After a 3 x 15 minutes wash in PBS-T, sections were incubated in rhodamine or FITC goat-anti-mouse/rabbit IgG/IGM (Pierce; 1:100 in 0.1% Triton X-100 in PBS) for one hour. After another wash in PBS for 5 minutes, coverslips were mounted onto microscope slides in anti-fade reagent (Citifluor Ltd) for microscopy and sealed around the edges using translucent nail varnish.

Antibody	Source	Reference
Anti-mouse Isl1/Isl2	Developmental Studies Hybridoma Bank (DSHB)	Park et al., 2002
Anti-mouse Glutamine synthetase	Chemicon International, Temecular, CA)	Peterson et al., 2001.
Anti- zebrafish *olig2*	CovaLab Inc.	In house (Dr. Huliang Li)
Anti-mouse GFAP	Sigma	Kawai et al., 2001

Table 2.3 List of antibodies used for immunohistochemistry

2.5.1 Combined BrdU immunolabelling and *in situ* hybridization

To label zebrafish cells in S phase of mitosis *in vivo*, 10μM BrdU (diluted in fish water) was added to the living fish and left to incorporate at 25°C for a standard 1 hour. Embryos were then washed and fixed, sectioned and subjected to *in situ* hybridization (as described in Section 2.4.2). The *in situ* hybridization reaction was stopped by washing in PBS for 30 minutes at room temperature. Sections were treated at room temperature, permeabilised for 15 min in 1% PBST. Six M HCl in 1% (v/v) PBS-T was then applied for thirty minutes at 37°C, followed by washes with PBS-T and 4% normal goat serum in 1% PBS-T for 30 minutes. Sections were incubated overnight at 4°C in monoclonal anti-BrdU, (hybridoma supernatent BU209 (Magaud et al., 1989)), diluted 1:4 in 0.1% (v/v) Triton X-100 in PBS. After a 3 x 15 minutes wash in PBS-T, sections were incubated in rhodamine goat-anti-mouse IgG (Pierce) 1:100 in 0.1% PBS-T for 30 minutes. After another wash in PBS for 5 minutes, coverslips were mounted onto microscope slides in anti-fade reagent (Agar Scientific) for microscopy and sealed around the edges using translucent nail varnish.

2.5.2 Hoescht staining

To identify all cell nuclei in sections, Hoescht bisbenzimide (clone number 33258, Sigma) was used. Sections were incubated in 0.1mg/ml Hoescht bisbenzimide (from 10mg/ml stock solution) in PBS for 5 minutes at room temperature before covering with a coverslip.

2.5.3 Detection of β-galactosidase

Whole zebrafish embryos were stained for β-galactosidase activity according to standard procedures. Embryos were dechorionated if required and fixed for 1-20 minutes (according to stage of development) at room temperature in 1% Formaldehyde, 0.2% Glutaraldehyde (GDA), 2mM $MgCl_2$, 5mM EGTA and 1X PBS. Fixed embryos were washed many times in Wash buffer (0.1 M Phosphate buffer pH 7.3, 2mM MgCl2, 0.1% Sodium deoxycholate, 0.02% NP40) at room temperature. Then, they were incubated in X-gal reaction buffer (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM MgCl2, 0.4% X-gal in PBS) for 2-4 hours at 30°C. Embryos were rinsed many

times in PBS until the solution no longer turned yellow and postfixed in 4% PFA. Staining was viewed under bright field optics for optimal detection. Embryos were sectioned to observed lacZ expression at cellular level.

2.5.4 Microscopy

Light and fluorescence was viewed under a Zeiss Axiophot microscope connected to a digital camera (Hamamatsu). Images were captured using simple PCI (Imaging Systems) imaging software. Images were manipulated where necessary with Adobe Photoshop software.

The main dissecting microscope used was a Zeiss Stemi SV11 (Carl Zeiss Ltd, Welyn Graden City, UK).

2.6 Electron microscopy

Larval zebrafish to be processed for electron microscopy were immersion fixed in 1% gluteraldehyde, 3% paraformaldehyde in 100mM sodium cacodylate buffer pH 7.2 overnight at 4^0C. After washing the embryos, they were incubated in a 1% osmium tetroxide solution, dehydrated and embedded in Epon. Ultra-thin sections were stained with uranyl-acetate and lead citrate. Zebrafish embryos were immersed in 2% paraformaldehyde / 1% gluteraldehyde in 100 mM cacodylate buffer pH 7.2. Sections were viewed and photographed using a Philips CM100 transmission electron microscope.

2.7 Zebrafish embryo injections DNA constructs

Injection needles were prepared by pulling filament-containing borosilicate glass capillaries (Word Precision Instruments 1B100F-4, outside diameter 1.0mm, inside diameter 0.75mm) with a vertical pipette puller (David Kopf Instruments) cutting the edge with tweezers and calibrating under the microscope with a millimetre ruler. Injection system consisted of a needle holder (Word Precision Instruments) carried by a 3-axis micromanipulator (Narishige) connected to nitrogen-filled tubing commanded by

a control panel (Word Precision Instruments) and triggered by a foot pedal. Zebrafish embryos were injected at 1 cell stage with a concentration of 30ng-70ng/μl of DNA in 100mM KCl containing 0.02% phenol red. Embryos were aligned on the side of a glass slide in a glass petri dish, with just enough embryo water to ensure hydration.

2.8 Bioinformatics and genomics

All manipulations of DNA sequences were performed with DNASTAR package. Protein alignments were performed with the clustal methods in MegAlign in this software. Zebrafish expressed sequence tags (ESTs) corresponding to orthologues of mouse or human genes were sought by name in the nucleotide databases and by probing the zebrafish EST database with a given mouse or human protein at http://www.ncbi.nlm.gov/BLAST/. When available ESTs were ordered (Integrated Molecular Analysis of Genomes (IMAGE) or Resource Centre/ Primary datatabase, (RZPD) to obtain DNA templates for riboprobe synthesis.

2.9 Statistical evaluation of data

In general, experiments were repeated on numerous occasions (more than four times) to replicate and validate both experimental procedures and results. For evaluation, axial sections were taken through the trunk of the zebrafish embryos. Representative sections were selected for the montages in the figures displayed throughout the chapters of this Thesis. Similar anatomical locations were used in all figures. Typically, 40 sections from 7 different embryos were analysed in every experiment and positive results were found in all cases.

In the future, it would seem appropriate to include quantitative analysis to demonstrate reliability and robustness of the experimental techniques to validate the data.

Chapter 3

Neural Precursor Domains in Zebrafish Ventral Spinal Cord and Emergence of Oligodendrocyte Progenitors

3.1 Introduction

The zebrafish spinal cord is a relatively simple structure providing a suitable system for studying vertebrate neural specification and differentiation. By 24hpf it contains non-neuronal floor plate cells ventrally, neuroepithelial cells bordering the central lumen, and roof plate cells dorsally. Zebrafish neuroepithelial cells are also proliferative neural precursors giving rise to different types of neurons and glia. As the number of cells increases, the zebrafish spinal cord also enlarges laterally and longitudinally (Lewis and Eisen, 2003).

The dorsal-ventral (D-V) patterning of the spinal cord is fairly well studied in the mouse (see Section 1.3); less is known about D-V patterning in zebrafish. In both fish and mammals Shh from cells of the notochord and floor plate at the ventral midline (Krauss et al., 1993) and Bmp signalling from the roof plate, pattern the spinal cord and specify distinct precursor domains along the D-V axis, each giving rise to different types of neurons (Appel et al., 2001; Lewis and Eisen, 2003). Zebrafish have two Shh orthologs, namely *tiggy-winkle hedgehog* (*twhh*) and *sonic hedgehog* (*shh*) (Zardoya et al., 1996; Ekker et al., 1995). Both *twhh* and *shh* are expressed in the ventral midline of the embryonic zebrafish neural tube and brain, *twhh* expression becomes limited to the neural tube, whereas *shh* is also expressed in the notochord (Ekker et al., 1995). The evolutionary pathway of ray-finned fish (of which zebrafish is an example) witnessed a genome duplication event after its divergence from the lobe-finned fish (including tetrapods). It is therefore unsurprising that duplicate copies of some genes, present as single copies in tetrapods, are found in zebrafish (Amores et al., 1998; Postlethwait et al., 1998).

In zebrafish as in mammals, Shh signalling is required for establishing the spatial regulation of other transcription factors expressed in neural progenitors located in distinct positions along the spinal cord D-V axis. For example, Shh signalling has been shown to be necessary for *nk2.2, olig2* and *nkx6.1* spatial regulation in specific regions in the ventral spinal cord (Barth and Wilson, 1995; Park et al., 2002; Lewis and Eisen, 2004).

In mammals, cross-repressive interactions between Class I and Class II transcription factors (Section 1.3) appear to refine and maintain different progenitor domains along the spinal cord. Some of the zebrafish homologues of these genes are beginning to be

understood in terms of this model. Expression of the basic helix-loop-helix transcription factor *olig2* is required for repression of *iro3* expression in the progenitor domain of ventral spinal cord in zebrafish (Lewis et al., 2005). Zebrafish *iro3* has a similar function to Irx3 in mammals (Briscoe et al 2000). It is induced in neural progenitors and prevents neural differentiation in the intermediate spinal cord domain (Lewis et al., 2005).

Our understanding and knowledge of transcription factors involved in neural tube patterning and neural differentiation in zebrafish is increasing, putting in place the tools necessary for a complete study of D-V patterning in the spinal cord. Two Nkx2.2 transcription factor has been found in zebrafish; *nkx2.2a* (*nk2.2* in Barth and Wilson, 1995) and *nkx2.2b* (Schafer et al., 2005). Similarly, the zebrafish genome encodes two *pax6*-related genes, *pax6.1* and *pax6.2*, that are expressed in overlapping domains along the D-V axis of the spinal cord and other specific expressing regions (including eye, dorsal diencephalon, hindbrain, and pancreas) in a pattern reminiscent of the pattern of Pax6 expression in the mouse embryo (Nornes et al., 1998; Macdonald et al., 1994). Other transcription factors such as Nkx and Dbx genes involved in mouse ventral neural tube patterning are also being characterised and investigated in the zebrafish neural tube (Cheesman et al., 2004). In general, the expression of most of these genes closely resemble the pattern found in higher vertebrates and this analogy leads to the prediction that the development of the spinal cord in zebrafish will be regulated in a very similar way to that seen in the mouse. However, the zebrafish spinal cord is smaller with many fewer cells than higher vertebrates.

As in mammals, the different progenitor domains along the D-V axis of the zebrafish spinal cord give rise to different types of neurons (Nguyen et al., 2000; Lewis and Eisen, 2003). Thus, Park et al (2002) have shown that motor neurons are generated from discrete progenitor domains located ventrally along the D-V axis of the neural precursor layer. This observation resembles the origin of motor neurons from the pMN domain in the ventral spinal cord in mouse (Jessell, 2000; Briscoe et al., 2001; Rowitch et al., 2002; Kessaris et al., 2001).

As in other vertebrates (birds and mammals), the zebrafish spinal cord contains other cell types in addition to neurons, including oligodendrocytes (Tomizawa et al., 2000; Brosamle

and Halpern, 2002; Park et al., 2002). Park et al. (2002) confirmed that zebrafish oligodendrocyte marker *olig2* identifies oligodendrocyte precursors in ventral spinal cord on both sides of the floor plate, the same region as motor neurons were produced. Their study showed that *olig2* is required for the production of oligodendrocytes and also the primary motor neurons. They described a transient expression of *olig2* mRNA since its expression does not persist after 72hpf, the latest time point they studied (Park et al., 2002). A more recent paper using a transgenic zebrafish line, Tg [*olig2: EGFP*] (Shin et al., 2003) has shown that motor neurons, interneurons and oligodendrocytes can originate from individual ventral precursors cells that express *olig2:egfp* (Park et al., 2002). In addition, their studies blocking Shh signalling by cyclopamine (a Shh signalling inhibitor) showed that Shh signalling is temporally and spatially required for oligodendrocyte development in zebrafish (Park et al., 2004).

Sox10 is a transcription factor that identifies progenitor and mature oligodendrocytes in the CNS and other glial precursors in the higher vertebrate peripheral nervous system (PNS) (Kuhlbrodt et al., 1998; Stolt et al., 2002; Zhou et al., 2000). In zebrafish, *sox10* has also been shown to be expressed in oligodendrocyte precursors in the spinal cord from 48hpf and in Schwann cells in the lateral line (Dutton et al., 2001).

Despite recent data, the understanding of neural precursors and early oligodendrocyte specification in zebrafish is still in its infancy. This Chapter investigates the temporal and spatial expression pattern of specific neural and oligodendrocyte precursor molecular markers. The process of myelination by oligodendrocytes will be the topic of Chapter 4.

3.2 Results

I investigated the spatio-temporal gene expression patterns of *shh, nk2.2, olig1, olig2, sox10, iro3, pax6* in the zebrafish spinal cord using *in situ* hybridization. Clones of these genes or ESTs fragments were already available for use as molecular probes (see Section 2.4.1) except for *olig1* and *olig2*. Partial cDNA sequences for these two genes were cloned specifically for this study.

3.2.1 Identification of *olig1* and *olig2* zebrafish genes

BLAST searches of the zebrafish genomic database against mouse and/or human sequences for *olig2* identified fragments of zebrafish homologues. The sequences of these fragments were used to design primers to amplify the gene from zebrafish genomic DNA, in order to clone zebrafish *olig2*. Primers 5'- GCT TCA TCT CCT CCA GCG AGG -3' and 5'- AAA CTG AGA GCG CAC TGA ACC -3' generated a 458bp fragment (See Section 2.2). Since these experiments, a complete cDNA sequence for this gene has been published (Park et al., 2002).

To identify a genomic fragment of zebrafish *olig1* a translated BLAST search of the zebrafish database with mouse Olig1 protein was performed. The zebrafish *olig1* homologue was cloned by reverse transcript polymerase chain reaction (RT-PCR) from 2-3dpf zebrafish embryos total RNA by using the following primers: 5'-TCAGAATGCAGGCTGTGTCTGGTG- 3' and 5'-TCGGAAAACGCATGGCTGGATT-3' (*olig1* was cloned by Dr. H. Li). To define the extent of homology between *olig1* and other Olig family members, the *olig1* translation product was compared to other sequences. The predicted zebrafish protein was closely related to mouse Olig1 protein and was named *olig1* accordingly. To discover whether the DNA sequences of zebrafish *olig1* and *olig2* were evolutionarily conserved between species, sequence alignments were performed using the NCBI BLAST tool (Figure 3.1). The sequence alignment of *olig1*, *olig2* and other bHLH gene families in mice and human indicate a high level of homology. Comparing zebrafish and mice *olig1* protein sequence reveals homology of 63% amino acid identity in the bHLH domain.

3.2.2 Ventral neural tube patterning with early specification markers

To identify the expression of *shh* and neural precursor gene markers *nk2.2*, *olig1* and *olig2* I performed *in situ* hybridizations in zebrafish at early stages. Expression of these markers was analysed in 24hpf whole mount embryos and cross-sections of their trunk region (spinal cord) and in cross sections of spinal cord at 48hpf and hatching stage (72hpf), once neurons have differentiated and oligodendrocytes precursors are produced in zebrafish.

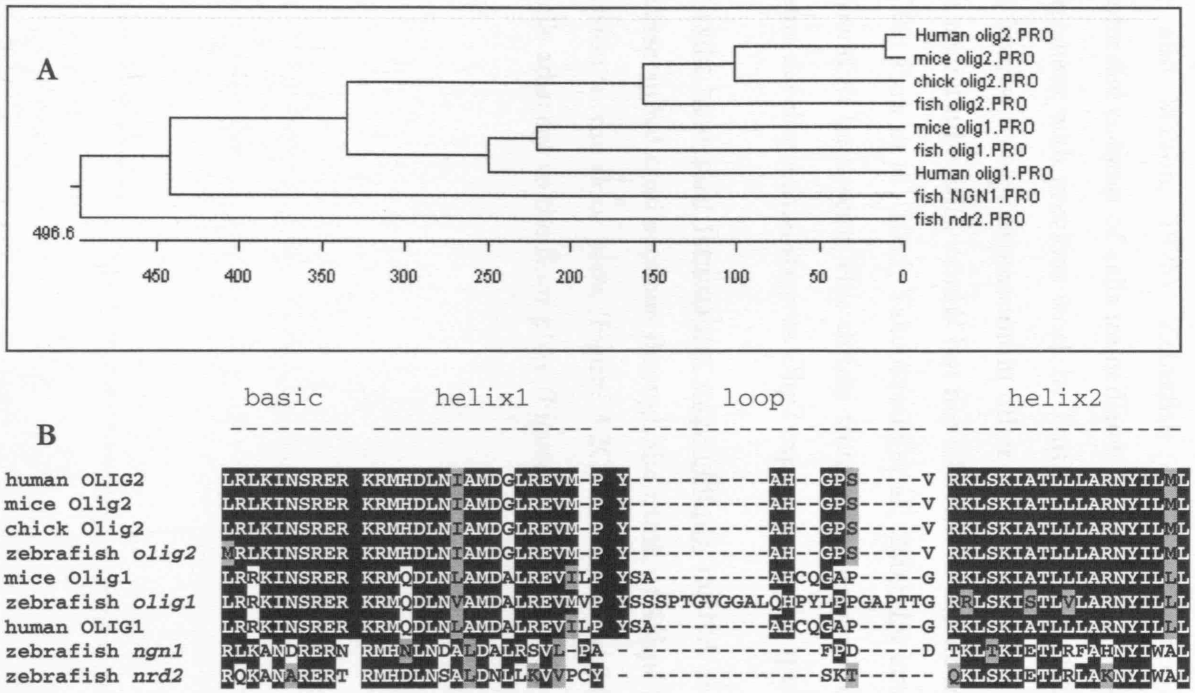


Figure 3.1 A. Phylogenetic tree showing the evolutionary relationship between zebrafish, human and mouse Olig1 and Olig transcription factors. Sequence alignments were performed using the ClustalW program provided with DNAstar, the dendrograr was generated using Bioedit programme. Two members, *neurogenin 1 (ngn1) neuroD(nrd)* of the bHLH superfamily was used as a outgroup. **B.** Alignments of the bHLH domains of a number of family members including *olig1*. Aminoacids that match are shaded black.

3.2.2.1 Pharyngula period

Twenty-four hours post fertilization (24hpf). RNA *in situ* hybridization analysis in whole mounted embryos showed *shh*, *nk2.2* and *olig2* staining in the brain and within the spinal cord in a thin stripe one or two cell wide along the trunk (Figure 3.2A-C). Consistent with the expression in other species such as mouse and chicken, the position of *shh* signal within the spinal cord in sections across the trunk of 24hpf embryos appeared in the notochord and medial floor plate (Figure 3.2D) (Krauss et al., 1993; Strahle et al., 1996; Barth and Wilson, 1995). Zebrafish *nk2.2* expression is observed in a narrow ventromedial column of cells immediately dorsal to the *shh*-expressing floor plate. This is in agreement with previous work by Barth and Wilson (1995) and Shafer (2005) (Figure 3.2E). Similar to *olig2* expression in other species, localization is in a rather broad cluster of the neural tube cells, ventral but not adjacent to the floor plate (Figure 3.2F) (Zhou et al., 2000; Park et al., 2002; Takebayashi et al., 2000; Lu et al., 2000). Expression of *olig1* was found to be absent. This differs from Olig1 expression in mouse, which appears at the same developmental stage as Olig2 expression in the spinal cord (Qi et al., 2001).

Forty-eight hour post fertilization stage (48hpf). mRNA *in situ* hybridization analysis in transverse spinal cord sections through the trunk of 48hpf zebrafish embryo showed *shh* expression in the floor plate (Figure 3.2G). Zebrafish *nk2.2* expression is restricted to a few cells adjacent to the floor plate (Figure 3.2H

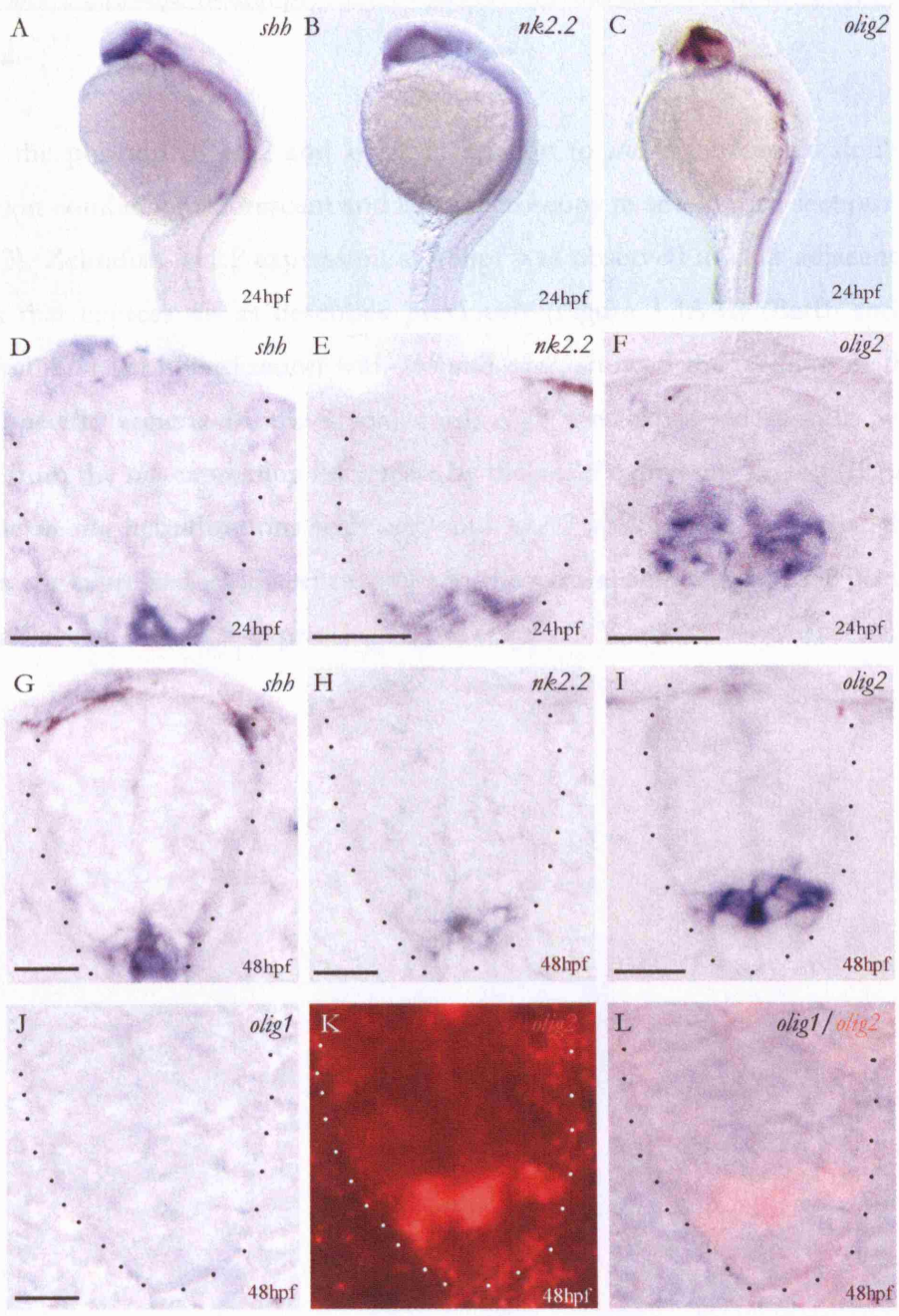


Figure 3.2 Developmental expression of *shh, nk2.2* and *olig2* in zebrafish spinal cord. Whole mount *in situ* hybridization showing the expression pattern of *shh, nk2.2* and *olig2* markers in zebrafish embryos at pharyngula stage (24hpf) (**A, B, C**). *Shh, nk2.2* and *olig2* staining is found in the ventral region of the trunk (arrows). Spinal cord sections of 24hpf showing the specific location of these genes within the spinal cord (**D, E, F**). At 48hpf, *shh* expression is in floor plate cells (**G**). *Nk2.2* (**H**) was expressed in neuroepithelial cells confined to a small ventral region just above the floor plate. *Olig2* (**I**) expression at this stage was found in the ventral spinal cord located in a small region dorsal to *nk2.2* expressing cells. *Olig1* was not expressed at any of these stages of development (**J**). A double *in situ* hybridization shows the presence of *olig2* (red, *in situ* hybridization (ISH)) transcript in a cluster of cells and the absence of *olig1* within the same section (**J-L**). Scale bars: 20µm

Zebrafish *nk2.2* and *olig2* neural precursor markers in relation to *shh* in the zebrafish spinal cord.

To locate the position of *olig2* and *nk2.2* in relation to *shh,* I performed double *in situ* hybridization combining fluorescent and light microscopy in spinal cord sections at 48hpf (Figure 3.3). Zebrafish *nk2.2* expression at 48hpf was observed in cells adjacent to floor plate cells that express *shh* as described previously (Figure 3.3A-C) (Barth and Wilson, 1995). Double *in situ* hybridization with *shh* and *olig2*, showed the staining of these two genes in specific regions of the spinal cord, *olig2* was expressed in cells which are separated from the *shh*-expressing floor plate by the *nk2.2* expressing region (Figure 3.3D-F). Double *in situ* hybridizations with *olig2* and *nk2.2* at 48hpf showed that these two transcripts are expressed in adjacent regions in the ventral spinal cord, *olig2* positive cells being found above the *nk2.2*-expressing region (Figure 3.3G-I).

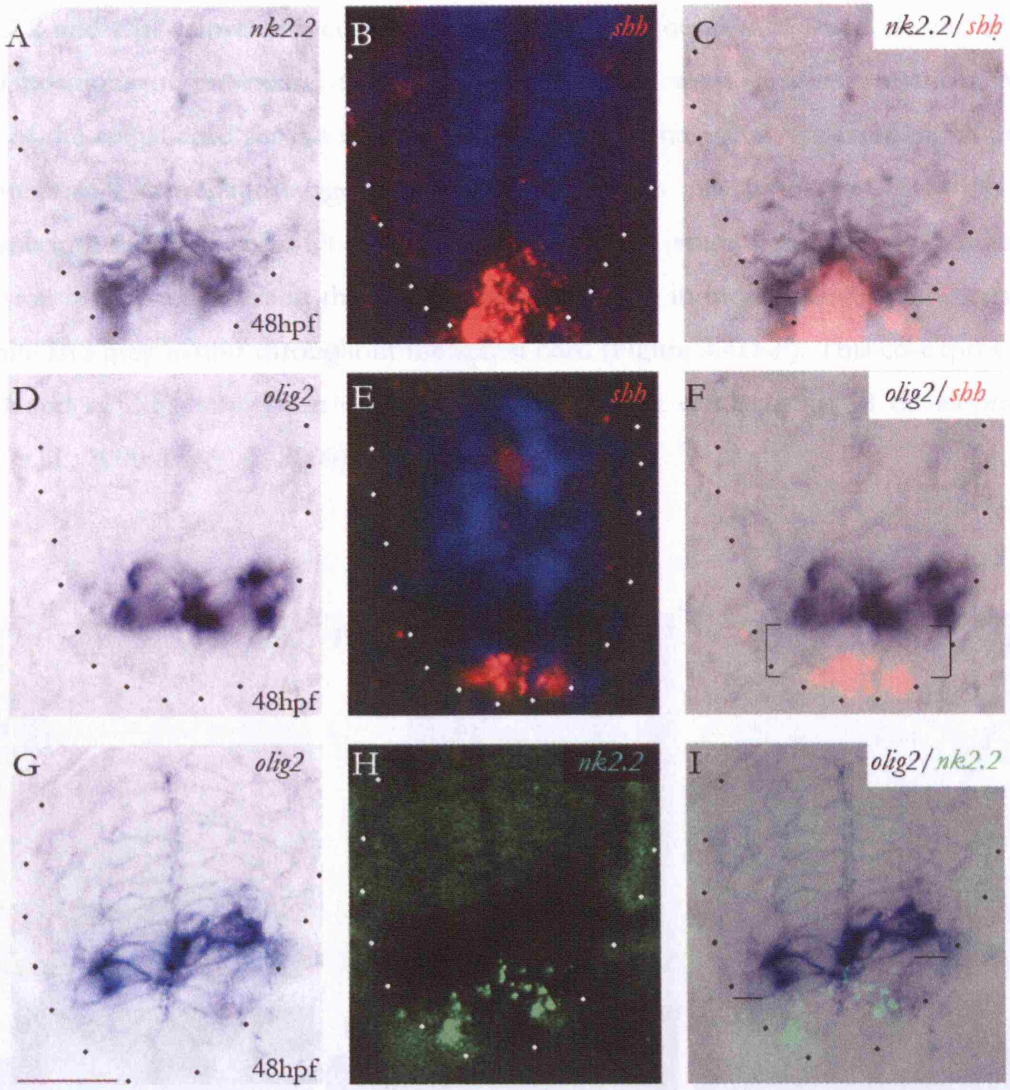


Figure 3.3 Double *in situ* hybridization for *shh* and *nk2.2* in transverse spinal cord sections of 48hpf zebrafish. Zebrafish *nk2.2* (brightfield) expression was seen adjacent to *shh* (red, ISH) floor plate-expressing cells (**A-C**). Zebrafish o*lig2* staining was seen in a cluster of cells close, but not adjacent to *shh* expressing cells (**D-E**). *Olig2* (brightfield) expression is located adjacent to the *nk2.2*- expressing (green, ISH) cluster of cells (**F-I**). Scale bars: 20µm.

3.2.2.2 Hatching period

Seventy two hours post-fertilization (72hpf). At this stage of development the expression pattern of neural precursor markers was found to be slightly different. Compared to expression in ventral clusters of cells in previous stages, *nk2.2* and *olig2* staining was

found in a number of cells dispersed throughout the spinal cord (Figure 3.4A-B). Some of the *nk2.2* and *olig2* expressing cells were, however, still found in a small cluster in the ventral positions of the neural tube. *Olig1* positive cells were observed in the ventral region of the spinal cord for the first time (Figure 3.4C) where they appeared in the same position as *olig2* expression suggesting that the two genes may be expressed within the same subpopulation of cells. Double *in situ* hybridization studies showed *olig1* and *olig2* expression in the same cells in the ventral region and also in individual cells dispersed in the white and grey matter throughout the spinal cord (Figure 3.4D-F). This co-expression of *olig1* and *olig2* has also been described in mammals at similar stage of development (Zhou et al., 2000; Lu et al., 2000).

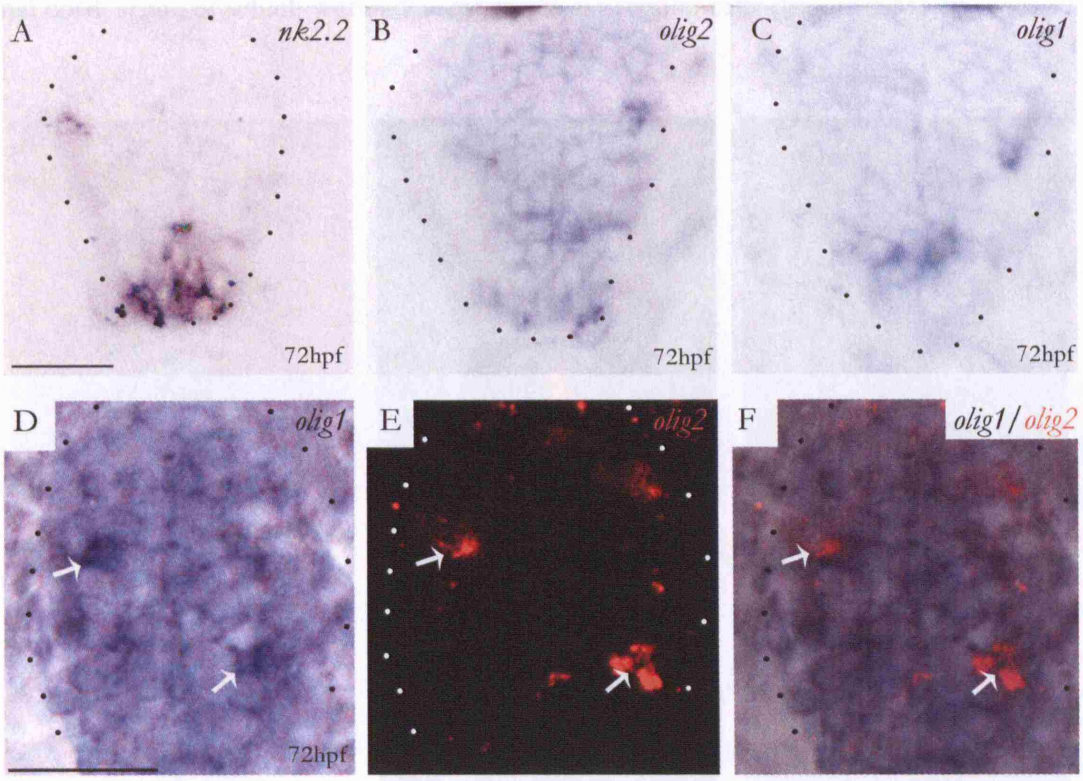


Figure 3.4 Expression of transcription factors *nk2.2*, *olig2* and *olig1* in transverse sections through a 72hpf zebrafish spinal cord. At 72 hpf *nk2.2* (**A**) and *olig2* (**B**) positive cells are found dispersed throughout the grey and white matter. At this stage some cells with both transcription factors *olig2* and *nk2.2* remain in the VZ. Zebrafish *olig1* (**C**) expressing cells are observed at similar location as *olig2* expressing region. Co-expression of *olig1* (blue) and *olig2* (red,ISH) in the same cells at 72hpf (**D-F**). Scale bars: 20μm

Double *in situ* hybridization of *nk2.2* and *olig2* in relation to the floor plate marker *shh* was studied in spinal cord sections (Figure 3.5). As previously described at 48hpf (see Figure 3.3G-I) *olig2* and *nk2.2* expressing cells occupy adjacent positions in the ventral neural tube, whereas *olig2* positive cells are separated from *shh* floor plate cells by the *nk2.2* positive domain. By 72hpf *nk2.2* expressing cells (Figure 3.5A) occupy a small region localized ventrally in the spinal cord as well as some cells scattered in dorsal regions. *Nk2.2* ventrally expressing cells occupy a very similar region in the spinal cord to *olig2*. Double *in situ* hybridization using *shh* and *nk2.2* probes shows that *nk2.2* expression continues to be adjacent to *shh* expression (Figure 3.5A-C).

The expression of *olig2* in relation to *shh* expression at this time of development shows that *olig2* staining was found in single dispersed cells and also in the ventral region of the spinal cord, some of which were adjacent to *shh* expressing cells (Figure 3.5D-F).

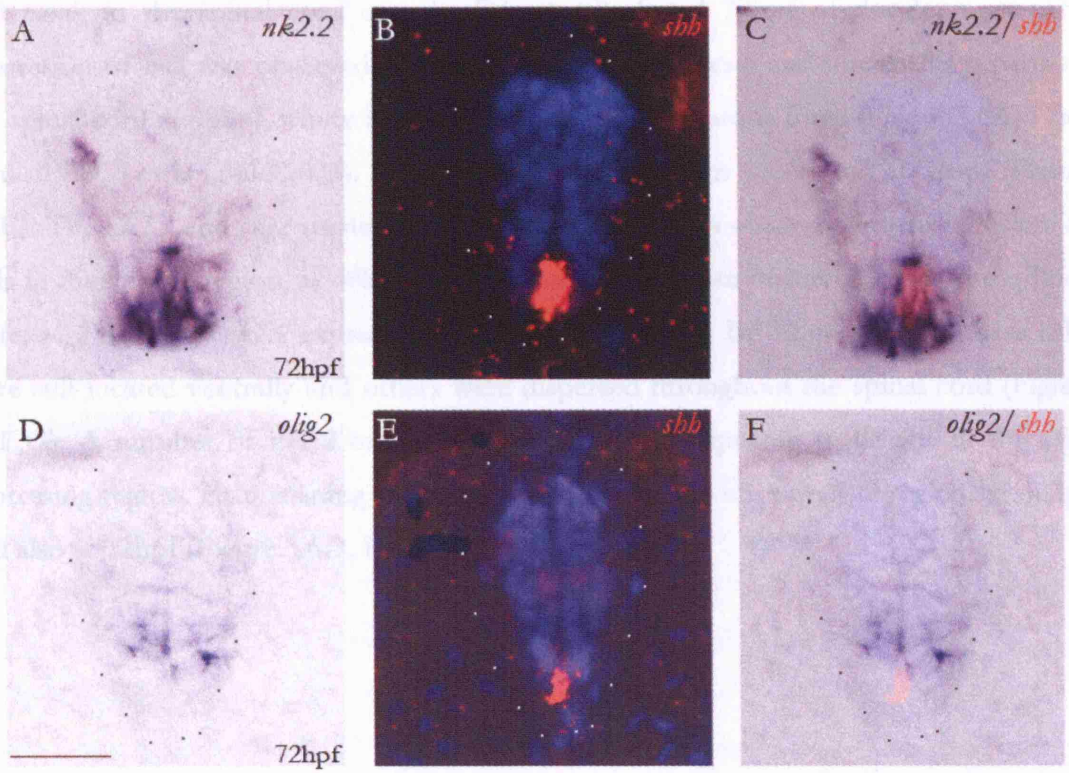
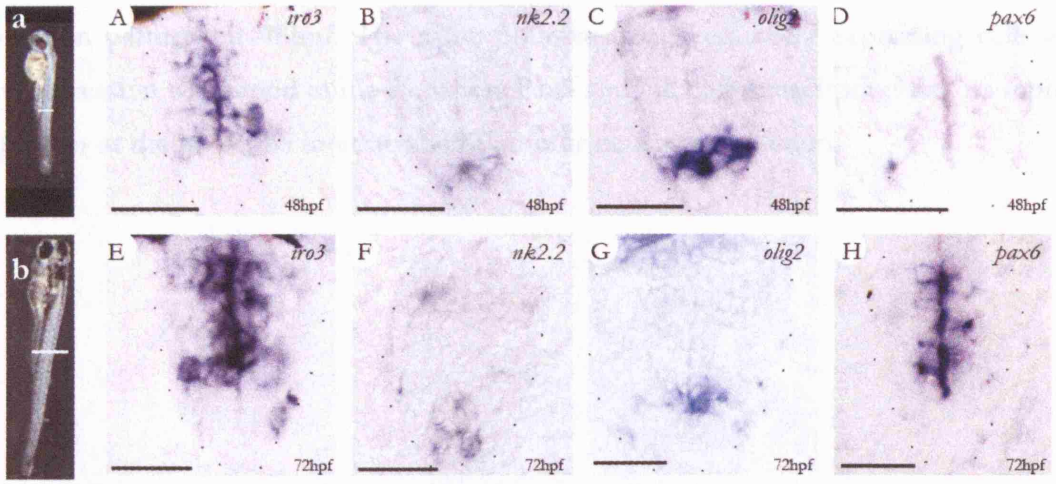


Figure 3.5 Double *in situ* hybridization for *shh* and *nk2.2* in transverse spinal cord sections of 48hpf zebrafish. Zebrafish *nk2.2* (brightfield) expression is seen adjacent to *shh* (red, ISH) floor plate expressing cells (**A-C**). Zebrafish *olig2* expression is located in a cluster of cells adjacent to *shh* expressing cells (**D-E**). Scale bars: 20μm.

In mice, several transcription factors (class I and class II) are expressed in specific progenitor cells defining the domains of the ventral developing spinal cord (Goulding et al., 1993; Ericson et al., 1997; Pierani et al., 1999). Among them, iroquois3 (Irx3*)* is a transcription factor homologous to Xenopus ziro3 (*iro3* in zebrafish). It is a class I protein repressed by Shh, expressed in the intermediate region of the spinal cord in mouse and its ventral limit defines the p2/pMN boundary by cross inhibition with Olig2 (class II protein, induced by Shh), which is expressed in the pMN domain in neural precursors of motor neurons. Pax6 is a class I homeodomain protein also repressed by Shh and expressed in the ventral region of the spinal cord establishing the p3/pMN boundary by cross inhibition with Nkx2.2 (class II protein, induced by Shh), which is expressed in the p3 domain in neural precursors of V3 interneurons (Briscoe et al., 2000; Ericson et al., 1997; Kessaris et al., 2001; Novitch et al., 2001).

Figure 3.6 shows the expression pattern of the four transcription factors: *iro3, nk2.2, olig2* and *pax6*, in the spinal cord of zebrafish at 48hpf and 72hpf of development. The expression of *iro3* was observed in a broad band in the dorsal and intermediate parts of the spinal cord at 48hpf, where interneurons and motor neurons form (Figure 3.6A) (Tan et al., 1999; Lewis et al., 2005). A similar expression pattern was found at 72hpf (Figure 3.6E). The *nk2.2* and *olig2* staining, as described before, was observed in small clusters of cells in the ventral region, at 48hpf. *Nk2.2* positive cells were observed close to the floor plate, *olig2* dorsal to *nk2.2* expressing cells (Figure 3.6B, C). By 72hpf some of these cells were still located ventrally and others were dispersed throughout the spinal cord (Figure 3.6F,G). A number of *nk2.2* expressing cells at 72hpf appeared to lie within the *olig2* expressing region. *Pax6* staining was localized in dorsal and intermediate regions at 48hpf and also at 72hpf (Figure 3.6D, H).



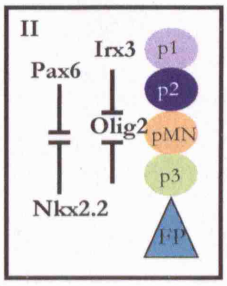
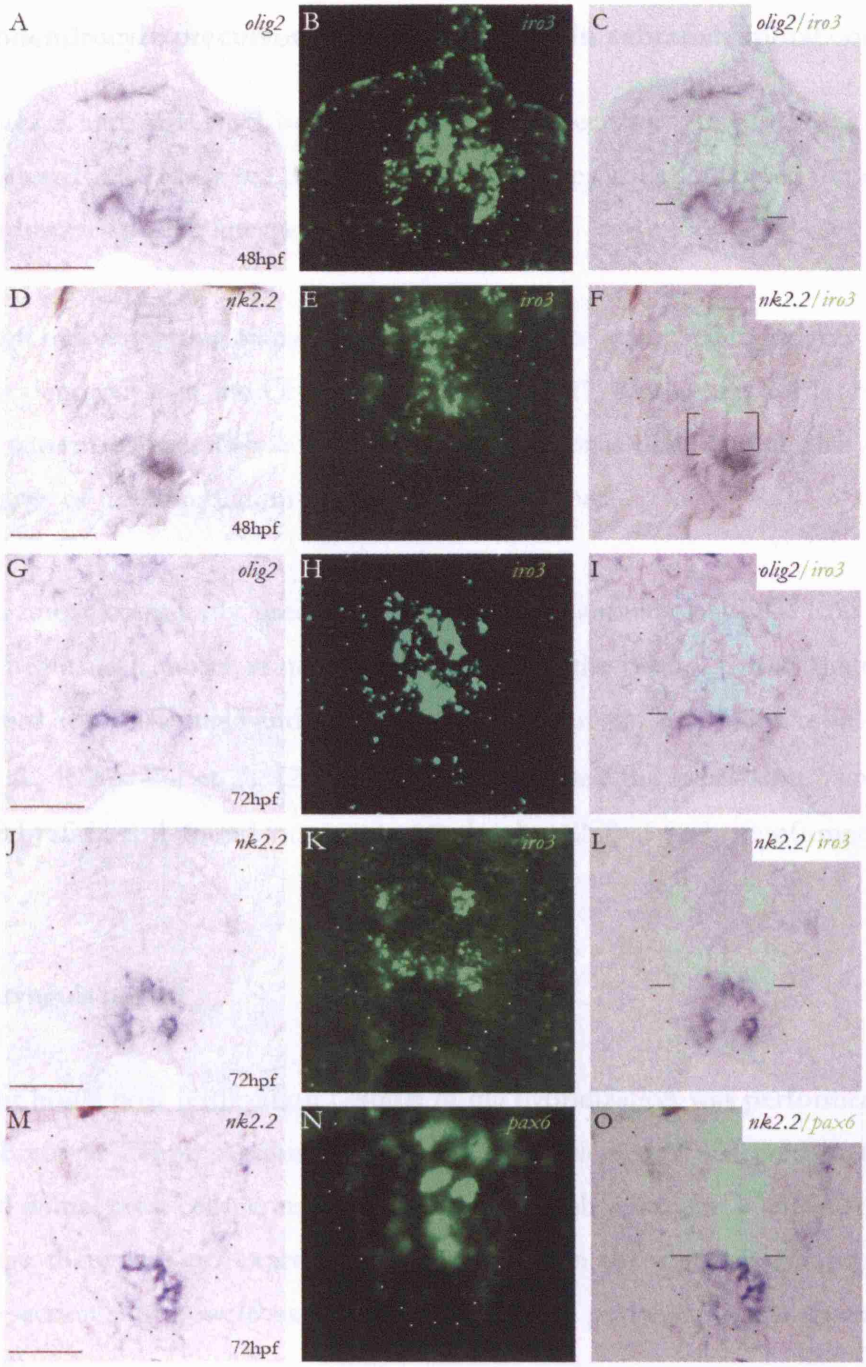


Figure 3.6 Expression pattern of ventral homeodomain transcription factors in spinal cord. Zebrafish *iro3* (**A,E**), *nk2.2* (B,F), *olig2* (**C,G**) and *pax6* (**D,H**) expression patterns in 48hpf (**A-D**) and 72 (**E-H**) hpf transverse spinal cord sections. **II.** Schematic representation of cross-interaction of homeodomain transcription factors in the ventral spinal cord. Photograph of a 48hpf (**a**) and 72hpf (**b**) zebrafish embryos. Scale bars: 20µm

To identify the position of *olig2* and *nk2.2* relative to *iro3* and *pax6*, I performed double *in situ* hybridization (Figure 3.7). The localization of *olig2* and *iro3* probes in the spinal cord at 48hpf showed expression in adjacent cells, delimiting a boundary between these two transcription factors (Figure 3.7A-C). Similar expression has been described at 24hpf by Lewis et al (2005). At 72hpf the expression pattern of these two markers remained largely unchanged. Cells expressing these markers were seen adjacent in the ventral region (Figure 3.7G-I). This is a very similar to expression patterns found in mammals where Olig2 and Irx3 transcription factors repress the expression of one another before and after motor neurons are produced. Double *in situ* hybridization in spinal cord cross sections at 48hpf with *nk2.2* and *iro3* probes located *nk2.2* ventrally, close to the floor plate, and *iro3* expression in intermediate regions revealing a region of non-expression between them, corresponding to the region where *olig2* expression might be expected (Figure 3.7D-F). By 72hpf the region expressing *nk2.2* appears to have expanded and within this region the *nk2.2* and *iro3* domains are now adjacent (Figure 3.7J-L). Similar results have been described in mouse where Nkx2.2 expression expands into the Olig2 domain, showing an overlap with Olig2 positive cells and adjacent to Irx3 expressing cells. Double *in situ* hybridization with *nk2.2* and *pax6* probes also shows the expression

of these markers in cells directly adjacent at 72hpf (Figure 3.7 M-O), similar to the expression patterns at 48hpf. The same boundary between *nk2.2* expressing cells and *pax6* expression was found in mouse where Pax6 and Nkx2.2 transcription factors repress each other at the pMN/p3 interface before motor neuron production.



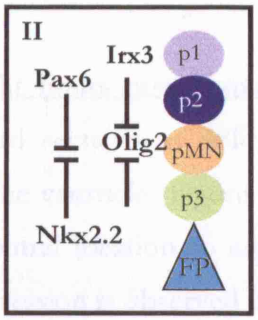


Figure 3.7 Expression pattern of ventral neuronal marker *iro3* and *pax6* in relation to oligodendrocyte transcripts *olig2* and *nk2.2* in zebrafish spinal cord. Double *in situ* hybridization localized the expression of *iro3* (green,ISH) in cells adjacent to *olig2* expression at 48hpf (**A-C**) and 72hpf (**G-I**). *Iro3* and *nk2.2* double *in situ*s hybridization showed a non expressing region at 48hpf (**D-F**) which was not found at 72hpf (**J-L**) where the two probes were expresed in adjacent cells. *Pax6* (green,ISH) is expressed in adjacent region 72hpf (**M-O**). Scale bars= 20µm **II**. Diagram representing ventral neuroepithelial domains and neuronal subpopulation markers in mouse spinal cord.

3.2.3 Oligodendrocyte precursor marker expression in zebrafish spinal cord

Zebrafish *nk2.2* and *olig2* label both neuroepithelial precursors and OLPs. To confirm that the scattered *olig1/olig2/nk2.2* positive cells could be OLPs I analysed the expression of oligodendrocyte specific lineage marker *sox10*.

Sox10, a high mobility group transcription factor has also been used to identify OLPs and mature oligodendrocyte in the CNS (Kuhlbrodt et al., 1998; Wegner, 2001). Herein, the expression pattern of zebrafish *sox10* transcription factor is described at pharyngula and hatching stages of development in the zebrafish spinal cord.

One of the most commonly used OLP markers in mammals is PDGFRα. PDGFRα expression begins in a subset of neuroepithelial cells in the ventral half of the embryonic rat spinal cord at E14 (Pringle and Richardson, 1993) and similarly in the mouse at E12.5 (Pringle et al., 1996). Lui et al., (2001) cloned and studied the expression of *pdgfrα* until 48hpf in zebrafish and found no expression in the CNS. I have confirmed this (see Chapter 5).

3.2.3.1 Pharyngula period

Twenty four hours post fertilization (24hpf) *In situ* hybridization was performed in whole mount embryos at 24hpf. At this stage, *sox10* expression was observed in the cranial ganglia, and neural crest cells, consistent with previous observations (Dutton et al., 2001). At this stage there was no expression of *sox10* within the spinal cord (Figure 3.8A). Transverse section shows *sox10* expression to be located peripherally, but absent centrally (Figure 3.8B)

Forty eight hours post fertilization (48hpf) *In situ* hybridization analysis in transverse spinal cord sections at 48hpf showed *sox10* mRNA expression in a cluster of cells flanking the ventricle (Figure 3.8C). The location of *sox10* expression at this stage was at similar ventral location to *olig2* positive cells at the same stage (Figure 3.2I). Zebrafish *sox10* expression is observed in a region dorsal to *nk2.2* expressing cells.

3.2.3.2 Hatching period

Seventy two hours post fertilization (72hpf). At this time of development, *sox10* mRNA expression was detected in a few scattered cells throughout the spinal cord, but mostly in cells occupying ventral and basolateral positions in the white matter (Figure 3.8D). The oligodendrocyte precursor markers *olig1*, *olig2* and *nk2.2* described previously were also found expressed in ventral and dispersed cells at this time of development (Figure 3.4).

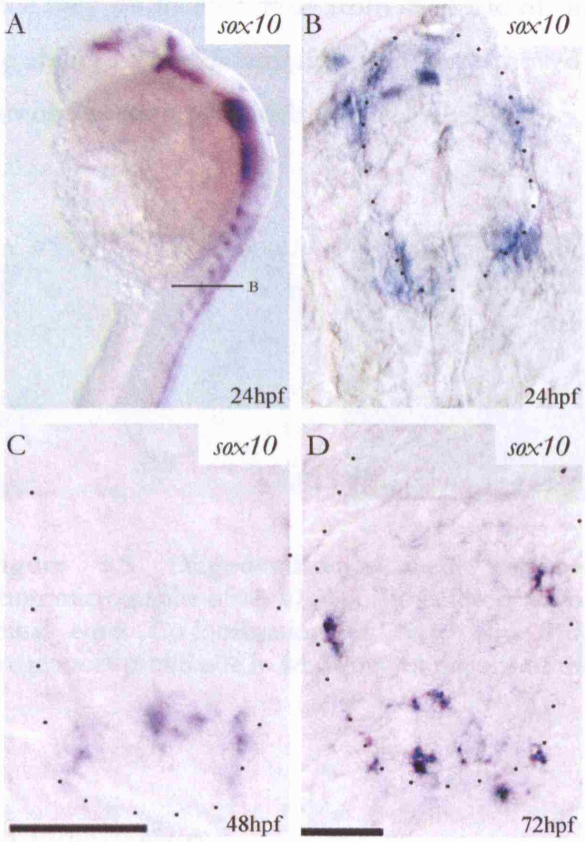


Figure 3.8 Oligodendrocyte specific marker *sox10* expression in zebrafish spinal cord.
Lateral (**A**) and dorsal (insert) views of whole mount zebrafish embryos at 24hpf. Cross-section of the 24hpf zebrafish trunk showing the expression pattern of *sox10* in the PNS. There was no expression of sox10 in the CNS at this stage of development (**B**). Zebrafish *sox10* expression in transverse spinal cord sections through a 48hpf embryo was found in cells in the ventral region of the spinal cord (**C**). By 72hpf *sox10* positive cells were found dispersed throughout the grey and white matter of the spinal cord (**D**). Sale bars 20μm.

One characteristic of OLPs in higher vertebrates is their ability to go through rapid proliferation, mainly during the immature progenitor stage previous to their differentiation (Bansal and Pfeiffer, 1992; Ono et al., 1995). The main proliferation of oligodendrocyte precursor cells appears to occur in the CNS grey and white matter rather than at their site of origin in the VZ. To test whether zebrafish spinal cord OLPs are proliferating cells, zebrafish embryos were incubated with bromodeoxyuridine (BrdU) and *in situ* hybridizations with *sox10* oligodendrocyte lineage marker were performed in spinal

cord sections, followed by immunofluorescence with anti-BrdU. Park et al (2002) have shown that *olig2* expressing cells in the VZ divide at 24hpf. Whether or not oligodendrocyte precursors have the ability to proliferate after they are found away from the VZ has not been reported in zebrafish.

Figure 3.9 shows the expression of oligodendrocyte marker *sox10* in single dispersed cells mainly in the white matter of a 96hpf zebrafish spinal cord. Some of these cells in the white matter were labelled with anti-BrdU antibody, indicating that these cells can divide once they are located away from their site of origin. In general, zebrafish OLPs maintain the ability to proliferate after they have moved from their site of origin, a characteristic also observed in mammals (Ono et al., 2001).

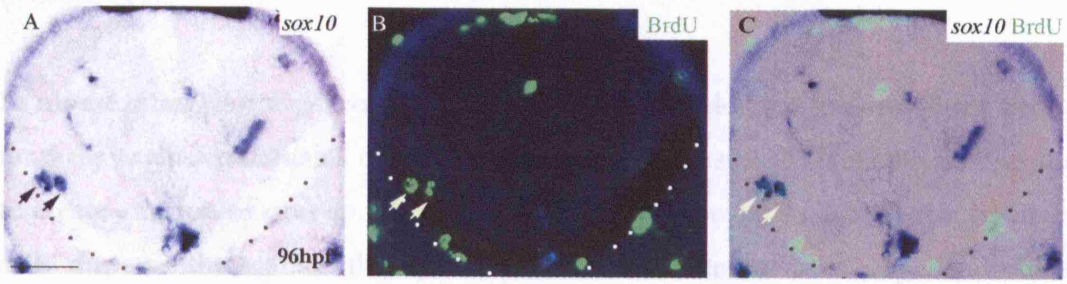


Figure 3.9 Oligodendrocyte cell proliferation in zebrafish spinal cord. Photomicrographs of *sox10* (**A**) oligodendrocyte marker and BrdU (**B**) mitotic marker in the spinal cord. Co-localization of *sox10* and BrdU (**C**) indicates that oligodendrocyte progenitors proliferate in white matter regions of the spinal cord.

3.3 Discussion

The main aim of these studies was to establish a baseline for zebrafish as a model system for the analysis of vertebrate oligodendrocyte development. Although the zebrafish spinal cord is a relatively simple structure, a full description of its development is not yet available. Most of the work in mapping the progress of oligodendrocyte development has been done in the mouse and chicken spinal cord. In order to establish the zebrafish as a suitable model, comparative studies with these species are necessary.

In this chapter I have analysed the developmental origin of oligodendrocytes in embryonic stages of zebrafish by studying in detail the temporal and spatial expression patterns of oligodendrocyte precursor markers (*olig1*, *olig2*, *nk2.2* and *sox10*) previously described for the mouse. Zebrafish o*lig1* and *olig2* were cloned for the purposes of the studies in this Thesis. Genes and neural precursor markers such as *shh*, *irx3*, and *pax6*, that are well characterised in the ventral spinal cord patterning in mouse were also investigated in order to obtain a better understanding of the spinal cord patterning in zebrafish. In general, zebrafish homologues of transcription factors that divide the ventral neuroepithelium of the spinal cord along the D-V axis in mouse were also found expressed in specific regions along the D-V axis of the zebrafish spinal cord. Together, these data are consistent with the notion that equivalent domains are defined in the zebrafish neural tube as well as in vertebrates that have been previously described (Briscoe et al., 2000).

In mouse, after motor neuron production in the pMN domain has ceased the previously mutually exclusive domains of Nkx2.2 and Olig2 expression shift relative to one another to create a region of overlap. Migratory Olig2/Nkx2.2 positive oligodendrocyte precursor cells disperse throughout the spinal cord and differentiate into oligodendrocytes. In zebrafish at 48hpf, *olig2* and *irx3* are expressed in adjacent domains until 72hpf, as described by Lewis et al (2005) at 24hpf. Then *nk2.2* expressing cells expand dorsally up to the ventral limit of *irx3* expression. Thus *nk2.2* expression expands dorsally in the zebrafish spinal cord at later stages, as it does in mice.

These studies using *in situ* hybridization demonstrate that the expression pattern of the oligodendrocyte precursor markers in zebrafish is similar to the expression of their homologues in higher vertebrates (birds and rodents). Zebrafish oligodendrocytes develop from ventrally derived progenitor cells identified by the early oligodendrocyte transcription factor *olig2* (Park et al., 2002) and *sox10* in a restricted domain in the ventral VZ dorsal to the *nk2.2* domain, which has been described in mammals (Takebayashi et al., 2002; Takebayashi et al., 2000; Zhou et al., 2000; Zhou et al., 2001) (Figure 3.10).

Nkx2.2 has been considered as another marker for oligodendrocyte in mouse and chicken (Fu et al., 2002; Qi et al., 2001; Soula et al., 2001; Zhou et al., 2001). This study describes the expression of *nk2.2* in relation to oligodendrocyte development in zebrafish. The

expression of *nk2.2* is initially detected in a more ventral domain of the VZ than *olig2/sox10* expressing cells in the zebrafish spinal cord. At later stages a number of *nk2.2* positive cells were found dispersed in the white and grey matter of the spinal cord; a similar expression to *olig1*, *olig2* and *sox10*. Some of the *nk2.2* expressing cells appeared to persist in the ventral region of the spinal cord.

As in higher vertebrates, zebrafish OLPs conserve the ability to go through proliferation during the immature progenitor stage prior to their differentiation. This proliferation occurs mainly away from the VZ.

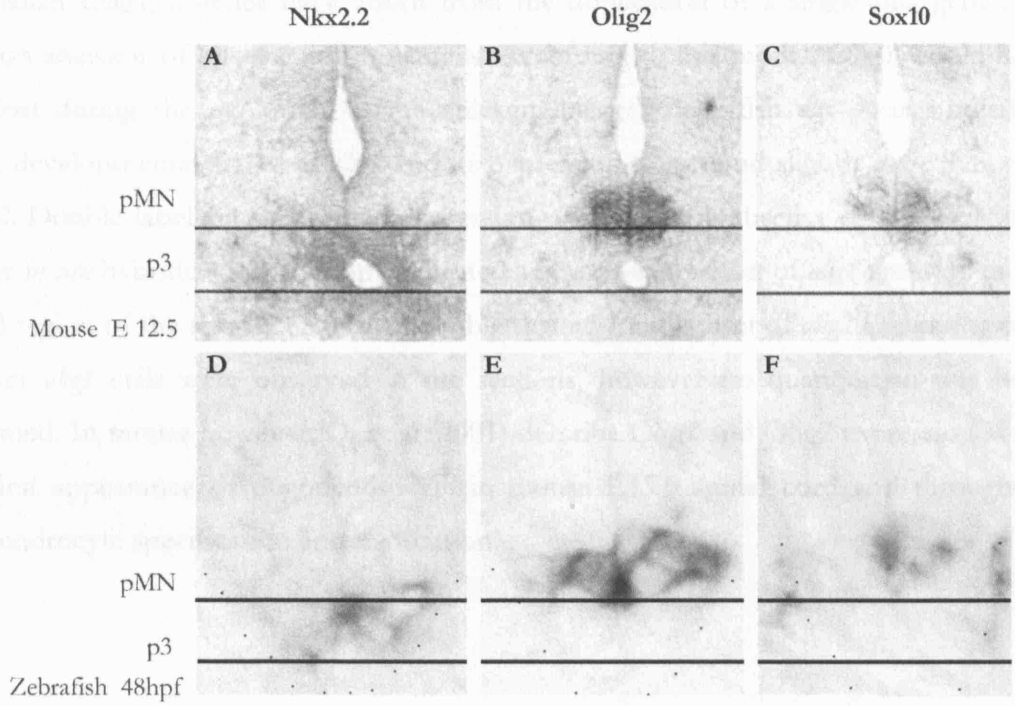


Figure 3.10 Expression of oligodendrocyte specific markers in mouse and zebrafish at similar developmental stages. Expression pattern of transcription factors Nkx2.2, Olig2 and Sox10 in transverse spinal cord sections through E13.5 mouse (**A-C**) and at a similar stage of development at 48hpf in zebrafish (D-F). In zebrafish, the expression patterns of precursor markers *nk2.2*, *olig2* and *sox10* resembles that observed in rodents. (**D-F**). Similar to the expression of Nkx2.2 in mouse (**A**), the expression in zerbrafish was localized above the floor plate (**D**). The expression of *olig2* and *sox10* mRNA transcripts localized at a similar location in a cluster of cells in the ventral spinal cord neuroepithelium, dorsally to the *nk2.2* transcript in mouse (**B,C**) and zebrafish (**E,F**). Data from Dr Nigel Pringle (A-C).

Some of my results differed from previously described findings. Park et al (2002), described transient expression of *olig2* disappearing by 72hpf. Here we showed that *olig2* was strongly expressed at hatching period 72hpf and is also found at stages beyond, as will be described in Chapter 4. Cells expressing *olig2* were found dispersed throughout the grey and white matter mimicking the pattern seen in mouse. The expression of *olig1* has not yet been described in zebrafish. Olig1 lies adjacent to Olig2 in the mammalian genome. As no homologue of Olig1 has been found in chicken, it has been suggested that Olig1 is derived from Olig2 via a recent gene event subsequent to the divergence of the birds and mammals (Zhou et al., 2000; Rowitch et al., 2002). The existence of a zebrafish *olig1* gene, which is also closely linked to *olig2* gene, indicates that both fish and mammalian Olig1/2 genes have arisen from the duplication of a single olig gene in a common ancestor of teleosts and mammals. According to this model, Olig1 would have been lost during the evolution of the chicken lineage. Zebrafish *olig1* was studied at similar developmental stages as *olig2* and its expression was found slightly later than that of *olig2*. Double labelling showed an expression of *olig2* in the absence of *olig1* at 48hpf. Double *in situ* hybridization at 72hpf indicated some co-expression of *olig1* and *olig2* in the ventral region of the spinal cord. It is possible that *olig1* is a subset of *olig2* expressing cells as fewer *olig1* cells were observed in the sections, however no quantitation was been performed. In mouse however, Qi et al (2001) describe Olig1 and Olig2 expression at the very first appearance of oligodendrocyte in mouse E13.5 spinal cord and throughout oligodendrocyte specification and maturation.

Chapter 4

Oligodendrocyte Differentiation in Zebrafish Spinal Cord

4.1 Introduction

The previous chapter described how OLPs are produced from the ventral neuroepithelium of the zebrafish spinal cord and then migrate to the periphery whilst continuing to divide (Figure 3.9). This is equivalent to the migration and proliferation characteristics of OLPs in mammals. This chapter will focus on the subsequent differentiation of the zebrafish OLPs into myelinating oligodendrocytes. In mammals, this differentiation follows the morphological transformation of the bipolar progenitors to oligodendrocytes with multiple processes (Chapter 1, 1.4.1.4). Specific oligodendrocyte molecular markers are expressed at different stages of this process. In mice, PDGFRα, Olig1, Olig2 and Sox10 are expressed in neuroepithelial precursors in the pMN domain with the expression of Sox10 and Olig2 persisting throughout the OLP and myelinating oligodendrocytes stages (Chapter 1). Nkx2.2 is also expressed in oligodendrocytes (Qi et al., 2001; Fu et al., 2002; Soula et al., 2001). PDGFRα expression is down regulated prior to myelination (Hall et al., 1996; Pringle and Richardson, 1993) and Olig1 protein located in the nucleus in neonatal mice translocates to the cytoplasm in the white matter during development to adulthood (Arnett et al., 2004). In the myelinating stage these cells acquire myelin proteins MBP and PLP (oligodendrocytes in the CNS) or MBP and P_0 (Schwann cells in PNS) (Section 1.4.1.7). The expression of all these oligodendrocyte lineage markers representing various stages of oligodendrocyte development persists throughout adulthood (Levison et al., 1999; LeVine and Goldman, 1988; Watanabe et al., 2004; Nishiyama et al., 2002).

In zebrafish, *olig2:egfp* transgenic fish have shown that OLP-*olig2-egfp* cells also appear to undergo a transformation from bipolar, elongated cells to fine cellular processes (Shin et al., 2003). This occurs around 72hpf in the white matter of ventral and peripheral regions of the spinal cord where *olig2*, *sox10* and *plp* molecular markers are regularly expressed (Chapter3, (Park et al., 2002). Whether zebrafish OLP markers continue to be expressed in myelinating oligodendrocytes or if proliferating precursors are present in the adult zebrafish is unknown. Bromsale et al, (2002) have characterised myelin protein markers (*mbp*, *plp* and p_0) in whole mounted embryos, describing the onset of myelination from 48hpf beginning in ventral regions and maturing in a rostro-caudal pattern, as occurs in mammals (Brosamle and Halpern, 2002). Table 4.1 illustrates the expression of these

myelin markers in the CNS and PNS of mouse and zebrafish. Bromsale et al., (2002) described p_0 exclusively in the CNS at early developmental stages, unlike in mammals where expression occurs only in the PNS, p_0 was later detected in the PNS of four-week-old zebrafish (Brosamle and Halpern, 2002). Electron microscopy studies in zebrafish spinal cord myelin have shown that the first glial cell processes are loosely wrapped around axons at about 72hpf. A few days later, at 7dpf many axons are surrounded by several layers of compacted myelin and extend more dorsally in the spinal cord. This shows that in general, morphological features of myelin in the CNS, such as spiral wrapping and multiple ensheathment by oligodendrocytes, are comparable to higher vertebrates.

		MBP	PLP	P_0
Mouse	CNS	+	+	-
	PNS	+	-	+
Zebrafish	CNS	+	+	+
	PNS	+	-	+

Table 4.1 Myelin protein expression in zebrafish and mammals. MBP and PLP have a similar expression in zebrafish and mammals. MBP is detected in the CNS and PNS in both species whilst PLP expression takes place in the CNS only. P_0, which is only expressed in the PNS of mammals, is detected exclusively in the CNS of the developing zebrafish. Abbreviations: Central Nervous System (CNS), Peripheral Nervous System (PNS), Myelin Basic Protein (MBP), Proteolipid Protein (PLP) and Myelin Protein Zero (P_0).

In vertebrates, including zebrafish, oligodendrocyte development and the process of myelination originates ventrally spreads caudally to the spinal cord and rostrally to the midbrain and optic nerve (Brosamle and Halpern, 2002; Small et al., 1987). In most mammals except rabbit, OLPs are unable to migrate out of the optic nerve into the retina keeping the axons in the optic nerve layer of the retina unmyelinated throughout life. It seems that this oligodendrocyte migration stopped at the lamina cribrosa of the optic nerve head (Small et al., 1987; Perry and Lund, 1990; ffrench-Constant, 1994). In opposition to this, in the chick, where the lamina cribrosa is absent (Ono et al., 1998; Arata and Nakayasu, 2003) ganglion cell axons in the retina are found myelinated and oligodendrocyte are able to go through stages of differentiation reaching maturity and

expressing myelin products (Nakazawa et al., 1993; Inoue et al., 1980; Villegas, 1960). In zebrafish, the myelin markers *mbp*, *plp* and p_0 have been described to reach the optic nerve, as in mammals, but not in retina itself (Brosamle and Halpern, 2002; Schweitzer et al., 2003). Teleosts lack the lamina cribrosa (Fujita et al., 2000), so it is expected to find expression of oligodendrocyte and myelin markers in the retina.

This chapter presents an investigation of the spatial and temporal expression of oligodendrocyte lineage markers (*olig1, olig2, sox10* and *nk2.2)* together with myelin protein markers (*mbp*, *plp* and p_0) in the spinal cord of larval and adult zebrafish. To determine whether these cells differentiate into myelinating oligodendrocytes I used double *in situ* hybridization to look for co-localization with the myelin marker *mbp*. The expression of these markers is also described in the retina.

In addition to characterising the gene expression and morphological changes associated with myelination in wild type fish, I also present an investigation of myelin development in the *otter* mutant, which was isolated in a screen for neurological mutants with defects in *mbp* expression (Kazakova et al., 2005). Electron microscopy was used to study the ultrastructure of the ensheathed CNS axons.

4.2 Results

4.2.1 Myelin specific gene expression in zebrafish spinal cord

I characterised the expression of three major myelin-specific proteins (*mbp*, *plp* and p_0) in the spinal cord of zebrafish from the early appearance of myelin at hatching (72hpf) until later stages (larval (7dpf), juvenile, (one-month-old) and adult stage (six-months-old). Figure 4.3 shows the expression pattern of the myelin protein markers, *mbp*, *plp* and p_0, using *in situ* hybridization in spinal cord sections at 72hpf and one-month-old zebrafish. At 72hpf (Figure 4.1A,C,E) all three markers were expressed in single cells in a similar stereotypical pattern, reminiscent of dorsal and ventral root exist sites. Occasionally a small number of marked cells were present in the grey matter. By one month (Figure 4.1B,D,F) the number of cells expressing myelin markers in the spinal cord had increased, although positive cells were still predominantly located at the same dorsal and ventral root exist stereotypical sites as in larval fish stage.

The location of individual cells that express these myelin markers through different stages is very similar to that observed using the oligodendrocyte lineage markers *olig1*, *olig2*, *sox10* and *nk2.2*.

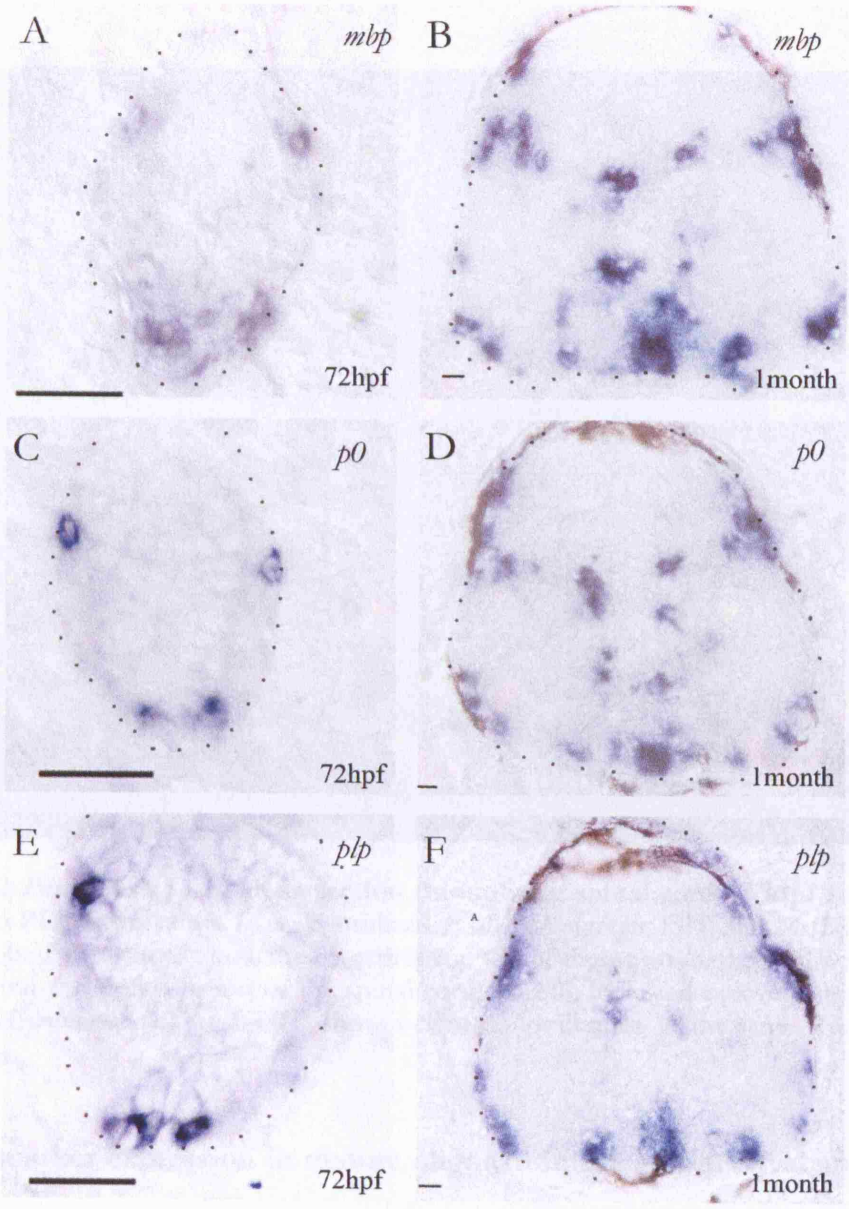


Figure 4.1 Myelin specific gene expression markers in larval and young adult zebrafish. Transverse sections through the spinal cord of zebrafish embryos at 72hpf (**A**,**C**,**E**) and one-month-old zebrafish (**B**,**D**,**F**) hybridised *in situ* with antisense for *mbp* (**A**,**B**), p_0 (**C**,**D**) and *plp* (**E**,**F**). Individual expressing cells were detected mainly in the white matter with additional scattered cells in the grey matter. Scale bars: 20μm

I also performed double *in situ* hybridizations with p_0 and the two main CNS myelin markers *mbp* and *plp* to determine co-expression in the zebrafish CNS. *Plp* and p_0 are reportedly expressed in the same cells in amphibia (Yoshida and Colman, 1996). Double *in situs* performed with *plp* and p_0 in a 7dpf zebrafish spinal cord showed co-expression in most, if not all, the expressing cells (Figure 4.2A-C). P_0 also showed a close co-localization with *mbp* expression within the same cells in the spinal CNS (Figure 4.2D-F).

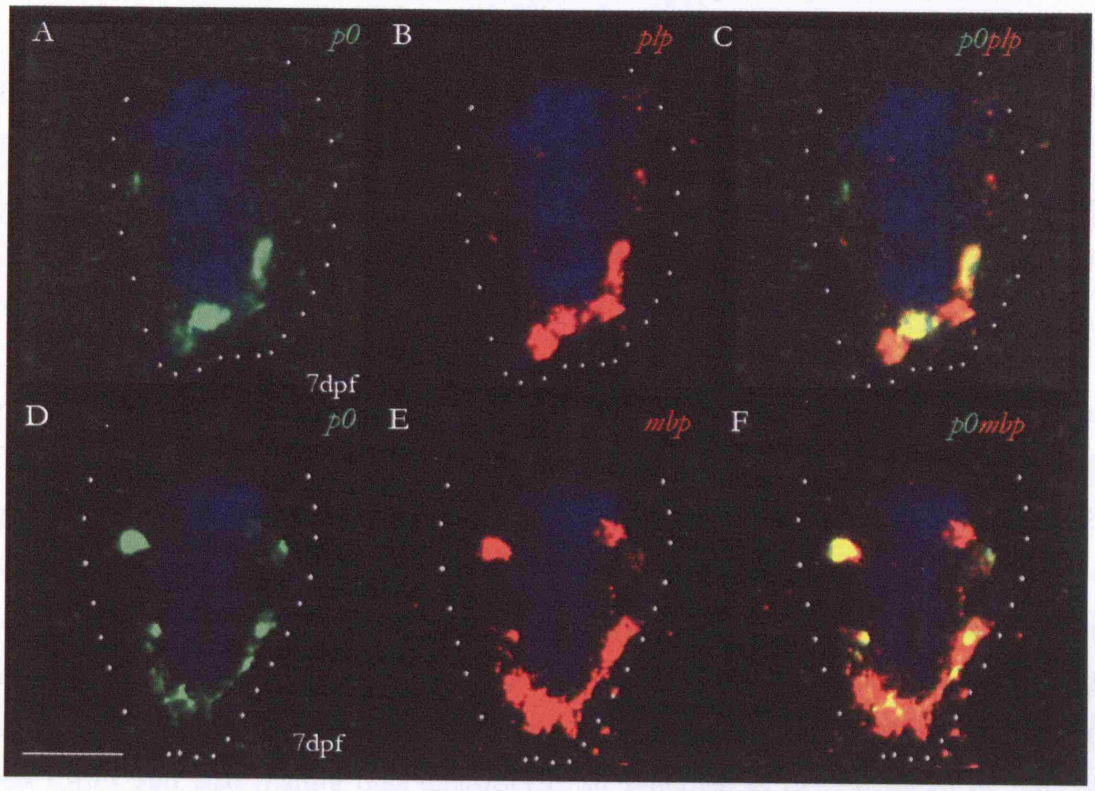


Figure 4.2 P_0 expression in cross section through the spinal cord of 7dpf zebrafish in relation to PLP expression. *In situ* hybridization of p_0 (**A**) (green, ISH) and *plp* (**B**) (red, ISH) on 7dpf zebrafish sections show the co-expression (**C**) of these two markers in the same cells in the ventral and dorsal region of the spinal cord in CNS, indicated by white arrows. p_0 (**D**) (green, ISH) and *mbp* (**E**) (red, ISH) show a close co-localization in the same cells (**F**). Scale bars: 20um

4.2.2 OLP marker expression in mature oligodendrocytes from larval and juvenile zebrafish

In situ hybridization at 7dpf (Fig 4.3 A, D, G, J) and one-month-old (Fig 4.4 A, D, G, J) in zebrafish spinal cord cross-sections showed *nk2.2, olig2, olig1* and *sox10* mRNA expression in cells distributed throughout the grey and mainly white matter of the spinal cord. They were detected in similar positions to those observed at 72hpf; that is, with cells located

mainly in a very ventral and latero-dorsal region of the spinal cord. The number of expressing cells in the spinal cord sections at 7dpf was seen to slightly increase for all markers, in comparison to 72hpf of development (Figure 4.1).

At these stages (7dpf, and one-month-old zebrafish) some *nk2.2* expressing cells persisted in the ventral VZ cluster (Figure 4.3A, 4.4A) whereas *olig2*, *olig1* and *sox10* expressing cells were only seen in scattered cells in ventral and dorsal-peripheral parts of the spinal cord. In chicken and rodents at similar stage, these markers including Nkx2.2 are found dispersed completely from the neuroepithelium in single cells throughout the parenchyma, without preference for dorsal or ventral, anterior or posterior (Fu et al., 2002; Qi et al., 2001). I also studied the expression pattern of *nk2.2*, *olig2* and *sox10* molecular markers in six-month-old zebrafish and in general observed a similar expression pattern to that in one-month-old fish.

To determine whether zebrafish OLPs markers continued to be expressed in differentiating oligodendrocytes I performed double *in situ* hybridization on larval (7dpf, Figure 4.3), and juvenile (one-month-old, Figure 4.4) spinal cord sections with *nk2.2*, *olig2*, and *sox10* and the myelin marker *mbp*. At 7dpf and one- month-old, a small number of *nk2.2* positive cells co-expressed the myelin marker *mbp* (Figure 4.3, 4.4A-C), which labels cell bodies and processes. *Olig2* positive cells were observed co-localizing with the *mbp* marker (Figure 4.3, 4.4D-F). Some *olig2* and *nk2.2* positive cells were *mbp* negative. *Sox10* labels OLPs and also mature oligodendrocytes in zebrafish as demonstrated by the co-localization with almost all *mbp* positive cells (Figure 4.3,4.4 G-I). Most *mbp* expressing cells, which were also positive for OLP marker expression, were located in the white matter of the spinal cord. By contrast cells that were *nk2.2/olig2/sox10* positive but *mbp* negative were mostly located in the grey matter suggesting that these *mbp* negative cells could represent immature progenitors as seen in mammals (Watanabe et al., 2002; Watanabe et al., 2004; Levison et al., 1999).

I also studied *olig1* expression with respect to the myelin marker *mbp*. Surprisingly, although *olig1* expression persists in larval and juvenile zebrafish I observed no *olig1* positive cells that co-labelled with *mbp* (Figure 4.3, 4.4J-L) or any other of the myelin markers such as *plp* or p_0 (data not shown). This suggests that in fish *olig1* is down

regulated in OLPs prior to differentiation. In mammals, *olig1* expression persists in mature oligodendrocytes although proteins translocate from the nucleus to the cytoplasm of myelinating oligodendrocytes (Arnett et al., 2004).

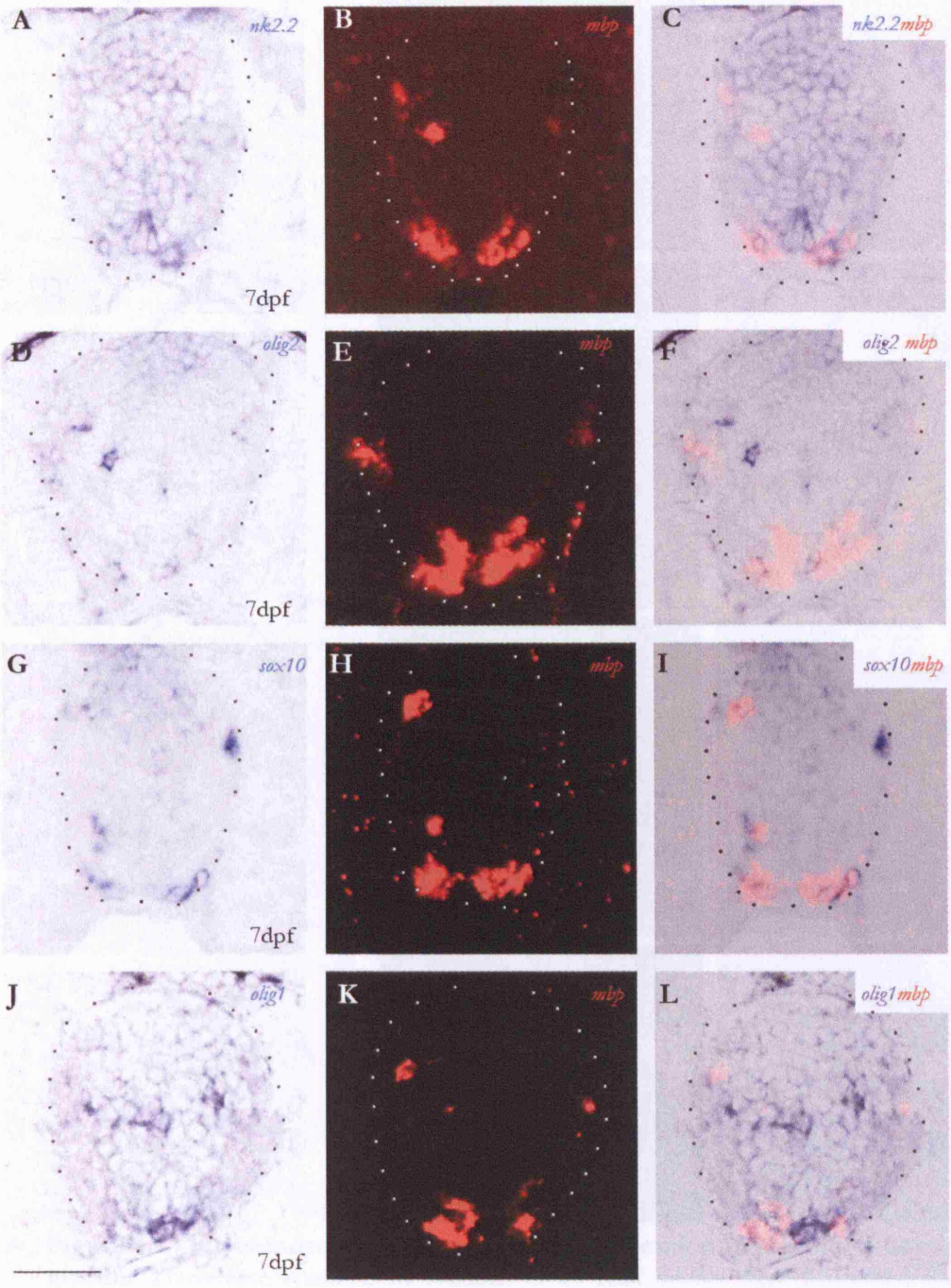


Figure 4.3 Oligodendrocyte lineage specific markers and myelin basic protein expression in 7dpf zebrafish. Transverse spinal cord sections from 7dpf were processed for double labelling *in situ* hybridization of *mbp* (red, ISH) with *olig2* (**A-C**), *nk2.2* (**D-F**), *sox10* (**G-I**) or *olig* (**J-L**) (blue) oligodendrocyte lineage specific markers. At 7dpf, myelin is most common in the ventral white matter of the spinal cord. A great number of *olig2* and *sox10* positive cells co-localize with myelin marker *mbp*. A smaller number of *nk2.2* cell also express *mbp*. *Olig1* positive cells do not co-localize with any *mbp* positive cells. Scale bars: 20μm.

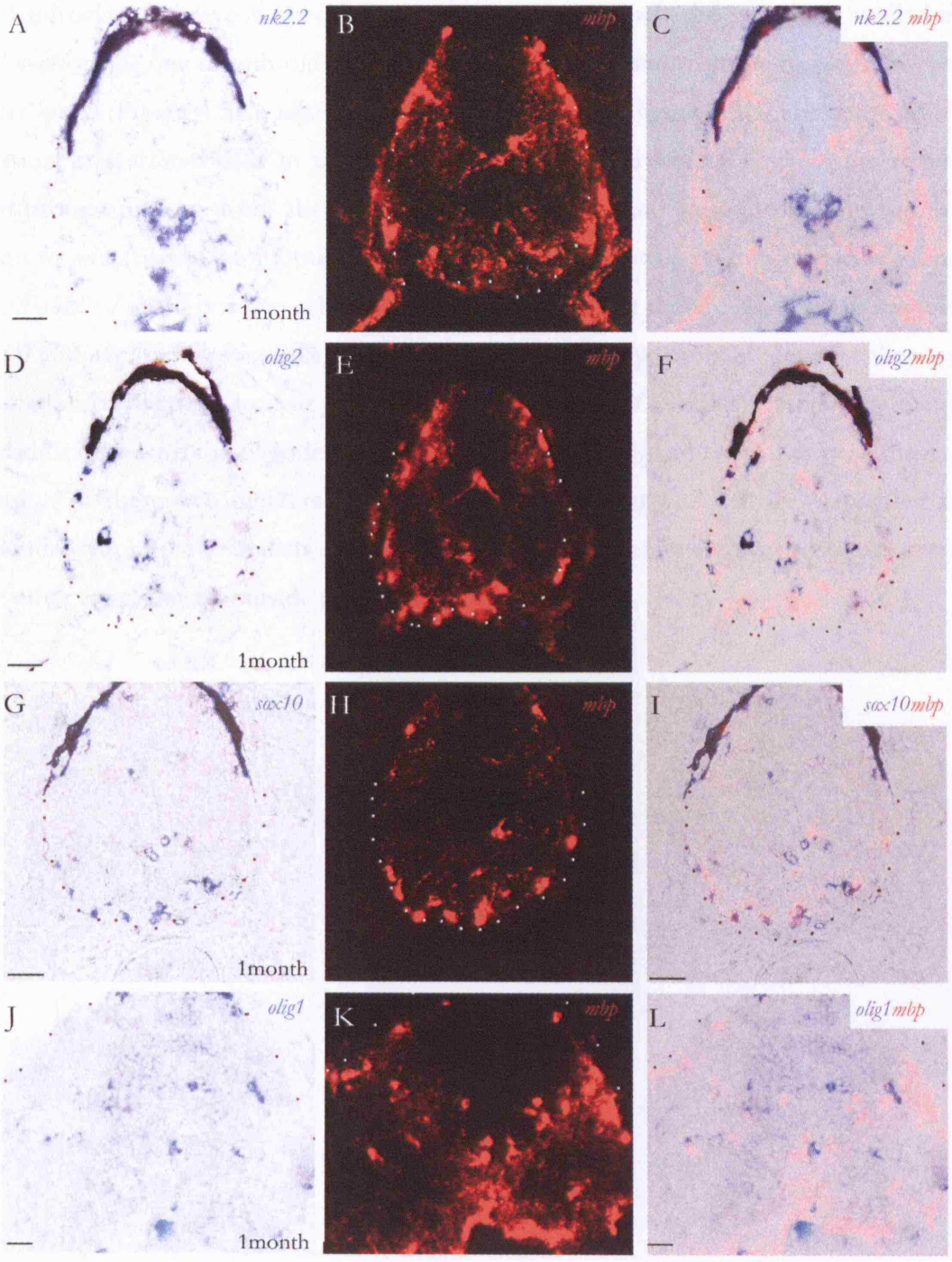


Figure 4.4 Oligodendrocyte lineage markers expression in juvenile cells expressing myelin. Transverse spinal cord sections from four weeks were processed for double labelling *in situ* hybridization of *mbp* (red, ISH) with *olig2* (**A-C**), *nk2.2* (**D-F**), *sox10* (**G-I**) or *olig1* (**J-L**) markers. Myelin is most common in the ventral white matter of the spinal cord at one-month-old. A great number of *olig2* and *sox10* positive cells co-localize with myelin marker *mbp*. A smaller number of *nk2.2* cell also express *mbp*. *Olig1* positive cells do not co-localize with any *mbp* (red, ISH) positive cells. Scale bars: 20μm.

4.2.3 Oligodendrocyte and myelin expression in the zebrafish retina

Oligodendrocyte and myelin specific markers studied in spinal cord were also studied in retinal sections of one-month-old zebrafish. *In situ* hybridization with the oligodendrocyte markers *sox10* (Figure 4.5A), *olig2* (Figure 4.5D) and *nk2.2* (Figure 4.5H) showed mRNA expression in scattered cells in and above the ganglion cell layer (GCL) of the retina. Myelin protein markers were also studied in this structure and were located in a similar position to *mbp* (Figure 4.5). Double *in situ* hybridization showed co-localization of some *mbp* and *olig2* or *sox10* positive cells indicating the maturity of these cells. A small number of *sox10* and *olig2* expressing cells did not co-localize with *mbp*, probably because they are a population of oligodendrocyte precursors cells in the adult zebrafish retina. Double *in situ* hybridization with the oligodendrocyte lineage markers *olig2* and *nk2.2* showed the co-expression of these two markers in the retina, meaning that *nk2.2* is also expressed in oligodendrocyte in the zebrafish retina. None of these markers were ever seen expressed in any other retinal layers outside the GCL.

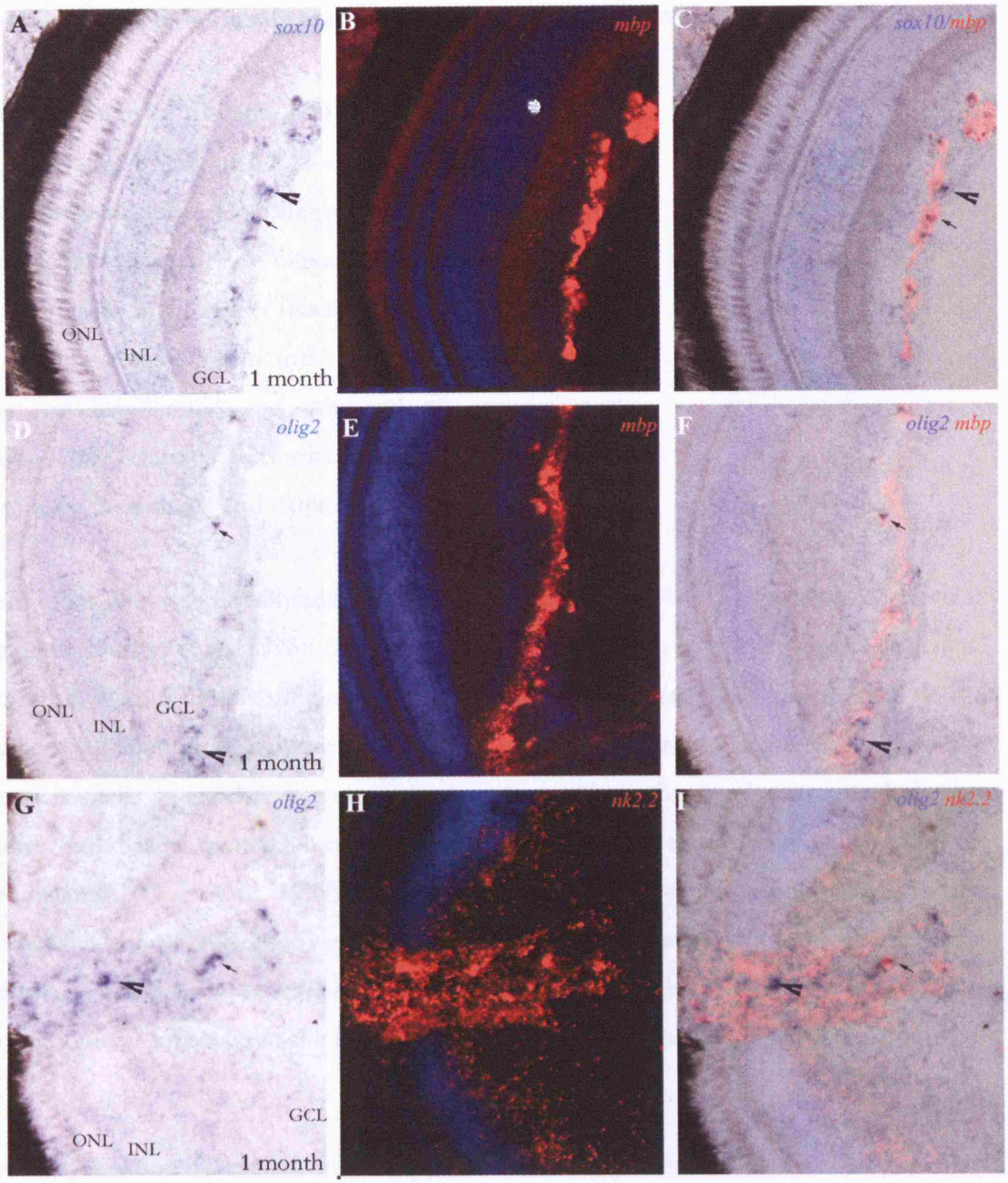


Figure 4.5 OLP and myelin expression in zebrafish retina. Double *in situ* hybridization showed the co-expression of the oligodendrocyte lineage markers *sox10* and *olig2* within the same cells as the myelin marker *mbp* (red, ISH)(**A-C**; **D-F**). Co-localization of *nk2.2* (red,ISH) and *olig2* expressing cells in the retina (**G-I**). *ONL*, outer nuclear layer, *INL*, Inner nuclear layer, *GCL* Ganglion cell layer.

4.2.4 Screening fish lines for mutants defective in glial development

A large number of mutants (45) that were held in the UCL fish facility were screened, by a member of the group, using *in situ* hybridization for effects on myelin basic protein marker (*mbp*) expression in oligodendrocytes in the CNS and Schwann cells in the PNS (Kazakova et al., 2006). Among the mutants screened, *otter* (ottA76B) showed an interesting effect on myelination and *mbp* and *plp* expression was lacking. *Otter* embryos were examined and selected prior to fixation by their shape and external appearance (Figure 4.6).

Figure 4.6 Photograph of *otter* mutant at 80hpf.

The mutant *otter* was initially identified from the large scale ENU mutagenesis screen in Tübingen (Haffter et al., 1996). *Otter* embryos have heart failures; display signals of general retardation and, like many mutants identified using ENU mutagenesis in zebrafish, do not survive until adulthood, tending to die in the early larval period around 96hpf. The *otter* mutant is allelic to another mutant, *motionless* which affects the developing catecholamine system, and has a reduced number of dopamine and catecholamine neurons in the hypothalamus (Guo et al., 1999). Visible morphological abnormalities are also apparent; the brain is mildly cyclopic, ventricles fail to properly inflate and blood circulation is disrupted. 48hpf *motionless* embryos do not or hardly respond to tactile stimuli. After 48hpf apoptosis increases in the lens and telencephalon and the fish dies around 96hpf.

4.2.4.1 Myelination characterization in the zebrafish *otter* mutant

Expression of the three myelin protein markers *mbp*, *plp* and p_0 was investigated in spinal cord cross-sections of wild type and *otter* mutant embryos for improved resolution of CNS cells. At 80hpf, the latest time possible to study in the *otter* mutant, there was an absence of expression of the *mbp* and *plp* myelin genes in the CNS of the spinal cord (Figure 4.7B,D) and also an absence of *mbp* in the lateral line, in comparison to the expression in the CNS of wild type embryos at the same time of development (Figure 4.7A,C). However, a very small number of p_0 expressing cells were observed in the CNS in the *otter* mutant in comparison to wild type embryos (Figure 4.7E, F).

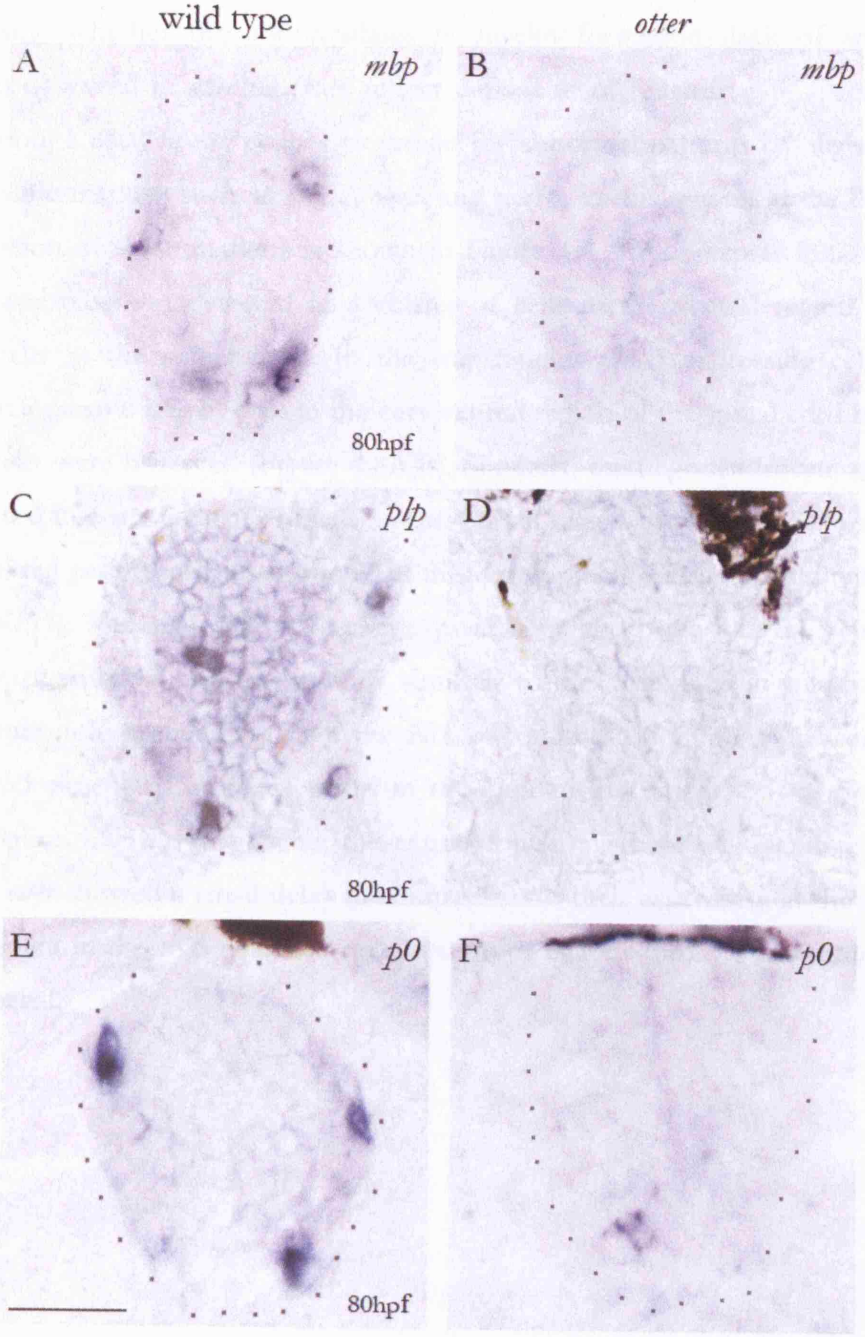


Figure 4.7 Myelin expressing genes in the *otter* mutant. *Mbp* (**B**) and *plp* (**D**) expression is absent in the *otter* mutant in comparison to their expression in wild-type embryos (**A, D**). A very small number of p_0 cells are found in the CNS in the *otter* mutant (**E**) versus wild type embryos (**F**). Scale bars: 20μm.

4.2.4.2 Neural and oligodendrocyte lineage characterization in the *otter* mutant

To determine whether the abnormalities in myelin formation (lack of *mbp* and *plp* expression) observed in *otter* mutants reflect defects in oligodendrocyte specification or differentiation, I used *in situ* probes to screen for abnormal patterns of oligodendrocyte lineage specific markers such as *nk2.2*, *olig2*, and *sox10*, in this mutant at the 80hpf stage. The expression of these markers is shown in Figure 4.8. *Nk2.2* expression at 80 hpf in wild type embryos was detected in a cluster of cells in the ventral region, and some scattered cells in the spinal cord. In the *otter* mutant *nk2.2* expressing cells are also observed in a narrow line of cells in the very ventral region of the spinal cord however no scattered cells were observed (Figure 4.8A,B). Similarly, the oligodendrocyte marker *olig2* was expressed in a small cluster of cells in the ventral region of the *otter* mutant compared to the scattered pattern of cells observed at this developmental stage in wild type embryos (Figure 4.8C,D). The expression of *sox10* expression is also present in the ventral region in *otter* mutant embryos and in the PNS, similarly to the expression in wild type (Figure 4.8E,F). Immunohistochemistry with the Isl1/Isl2 antibody for motor neurons showed normal development of motor neurons in this mutant in comparison to the wild type embryo (Figure 4.8H-I). In general, the expression of all these oligodendrocyte lineage markers in *otter* showed a small delay in comparison to their expression at the same stage of development in the wild type, but specification of oligodendrocyte and motor neurons appears normal.

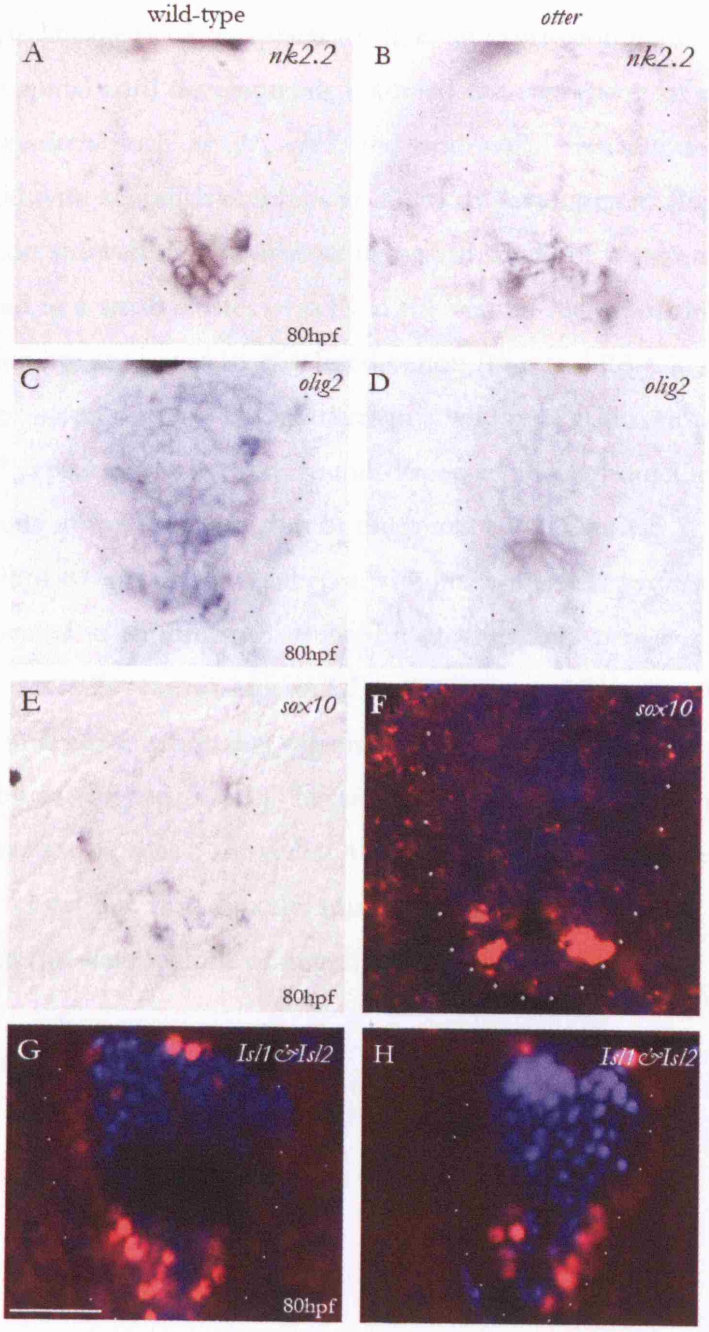


Figure 4.8 Oligodendrocyte lineage markers expression and motor neuron in wild type and *otter* spinal cord. Left panel shows wild-type expression, right panel the otter mutant expression. The expression of *nk2.2* (**A,B**), *olig2* (**C,D**) and s*ox10* (red, ISH) (**E, F**) are present in *otter* mutant but with a slightly different expression characteristic of an early stage such as 48hpf. The expression was slightly reduced in *otter* mutants in comparison to their wild type. ISL1/ISL2 antibody for motor neurons (red, Immunofluorescence, (IMG)) (**G,H**). Scale bars: 20μm

As oligodendrocyte lineage markers appear to show an expression pattern characteristic of an earlier stage in spinal cord development I studied the expression of early neural spinal cord patterning markers such as *shh*, *olig2*, *iro3* and *nk2.2* transcription factors in *otter* mutant versus wild type zebrafish embryos at 80hpf of development (Figure 4.9). Double *in situs* hybridization showed that *shh* was expressed in the floor plate and *olig2* expressing cells were observed in a small cluster of cells in the ventral region of the spinal cord. *Shh* and *olig2* expression are separated by a defined region (Figure 4.9A-C). This is in contrast to the relative expression of these two markers in a wild type at this time of development where single *olig2* expressing cells were found dispersed throughout the spinal cord and some cells remaining in the ventral region of the spinal cord (Figure 3.5). Double labelling experiments in 80hpf of *otter* mutant embryos with *iro3* and *nk2.2* probes showed that *iro3* expression was located in an intermediate region of the spinal cord leaving an unstained region with the ventrally expressing *nk2.2* cells (Figure 4.9D-F). By this time of development *iro3* and *nk2.2* expressing regions occupy adjacent regions in the spinal cord of wild type embryos (Figure 3.7J-L). In general the expression pattern of these four markers is characteristic of that observed at 48hpf (Figure 3.7D-F) where cells expression *shh* and *olig2* are close but not directly adjacent, *nk2.2* expression is ventral and *iro3* expression is in intermediate regions of neural tube.

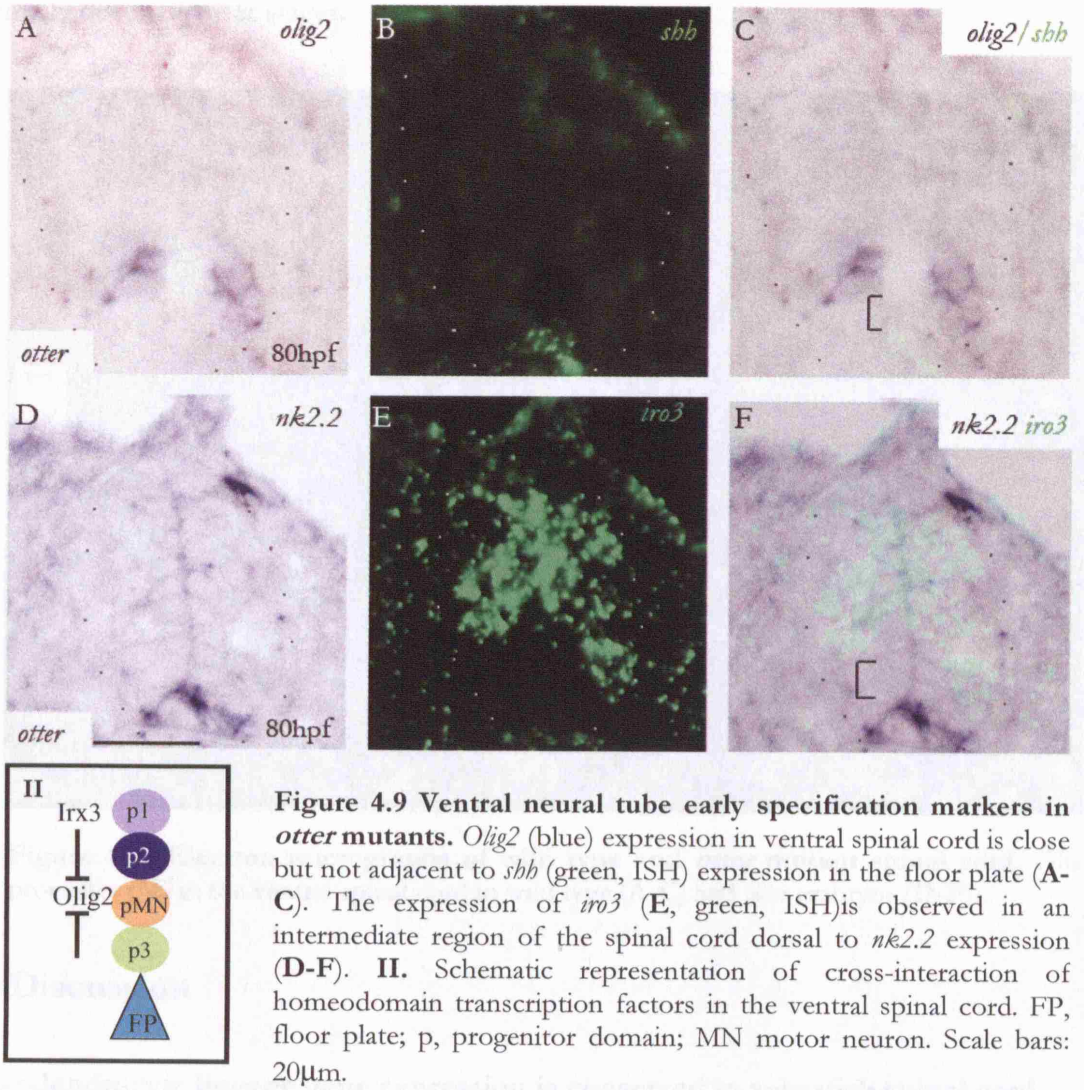


Figure 4.9 Ventral neural tube early specification markers in *otter* mutants. *Olig2* (blue) expression in ventral spinal cord is close but not adjacent to *shh* (green, ISH) expression in the floor plate (**A-C**). The expression of *iro3* (**E**, green, ISH)is observed in an intermediate region of the spinal cord dorsal to *nk2.2* expression (**D-F**). **II.** Schematic representation of cross-interaction of homeodomain transcription factors in the ventral spinal cord. FP, floor plate; p, progenitor domain; MN motor neuron. Scale bars: 20μm.

Characterization of the *otter* mutant with neuroepithelial and oligodendrocyte precursor markers shows very little effect on early oligodendrocyte specification in the zebrafish spinal cord. However, *otter* mutant embryos show an interesting phenotype of disrupted myelin expression with a lack of expression of *mbp* and *plp* markers in the CNS. A very small number of p_0 positive cells were observed in the spinal cord. Development of the myelin structure surrounding axons in the CNS was investigated in *otter* mutants using electron microscopy, (Figure 4.10). At 80hpf electron-micrographs of wild type and *otter* mutants from the ventral region of the spinal cord, where the processes of myelin usually begins, both showed the presence of axons (Figure 4.10) loosely wrapped by glial processes. Indeed, at this early stage the wrapped axons in the *otter* mutant appear looser in comparison to the wild-type embryos. However, it is not possible to know from this

data if this difference will persist in the development of zebrafish as compaction of myelin normally occurs at later stages.

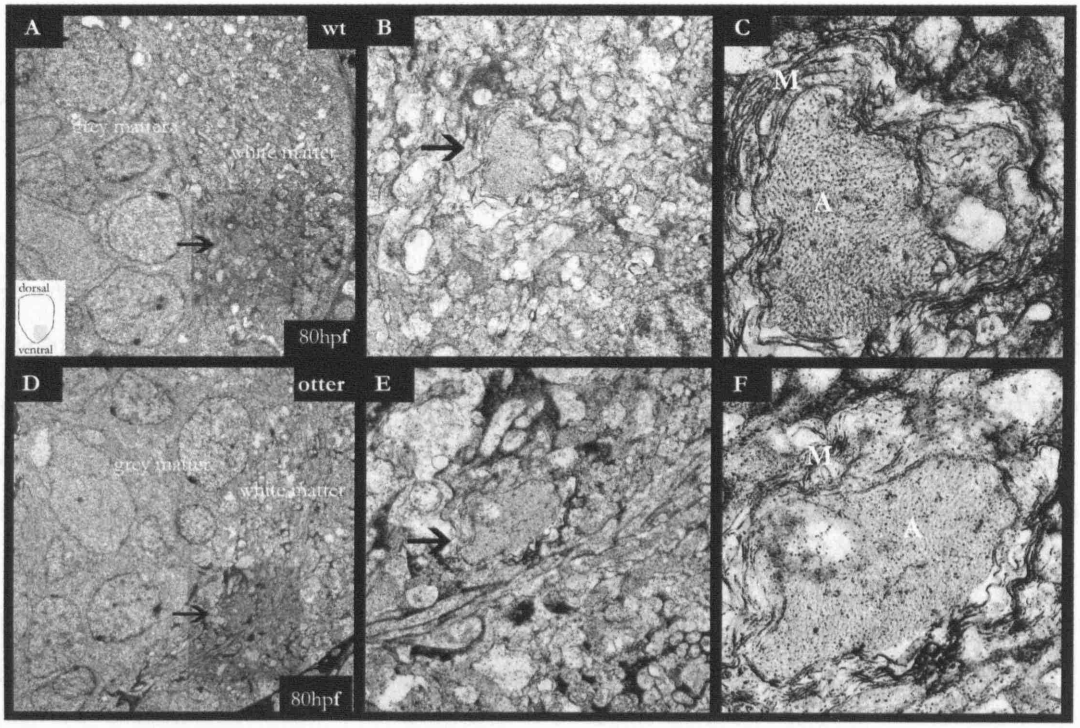


Figure 4.10 Electron micrographs of wild type and *otter* mutant spinal cord. Glial processes (M) in the ventral spinal cord in wild type (**A-C**) and *otter* embryos (**D-F**).

4.4 Discussion

Oligodendrocyte lineage gene expression is conserved in zebrafish spinal cord

Chapters 3 and 4 have described the spatial and temporal expression patterns of a range of markers for different stages of oligodendrocyte development in zebrafish.

These *in situ* hybridization studies generally demonstrate that gene expression during the specification and development of zebrafish follows a similar pattern to that observed in higher vertebrates (birds and rodents). In fish, as in mammals, the OLP lineage markers *olig2* and *sox10* are initially expressed in a restricted domain in the ventral VZ. Later expression of these markers (72hpf, 7dpf) can be observed in scattered cells throughout the grey matter as well as in cells at stereotypical ventral and lateral locations in the white matter (as described in chapter 3). The expression of these genes continues in scattered cell positions, which increase in number throughout the spinal cord until adult stages

(one-month, six-months). In the white matter, co-expression of OLP lineage markers with mature myelin proteins such as *mbp*, suggests that as in mammals, expression of *olig2* and *sox10* is maintained in differentiating oligodendrocytes.

Olig1 expression in zebrafish and mammals

As described in Chapter 3, it was surprising to find the *olig1* gene in the zebrafish, however, it seems that this expression differs to that of mammals where Olig1 is co-expressed with Olig2 throughout development. Both genes are expressed in the neuroepithelial precursors prior to gliogenesis, remaining active in OLPs that have begun to express glial cell-specific markers (Sox10/Pdgfrα), and can still be detected in differentiated oligodendrocytes in the slowly dividing oligodendrocyte progenitors cells of adult mammals. In fish, however, *olig1* expression appears to be restricted to the OLP stage of development. Transcripts are first detected in the ventral VZ 48-72hpf after *olig2* expression has become established. While *olig1* and *olig2* are co-expressed in OLPs, *olig1* is down regulated in differentiating oligodendrocytes.

The relatively restricted expression of *olig1* makes it a potentially useful OLP-specific marker in zebrafish. However, the functional significance of its down regulation in differentiated oligodendrocytes is unclear. In mammals, although Olig1 continues to be expressed in differentiated oligodendrocytes and adult oligodendrocyte precursors, it is excluded from the nucleus of these cells. Nuclear OLIG1 re-appears in regenerating precursors, which may be related to its role in myelin regeneration.

Myelin protein zero expression in zebrafish CNS

The myelin protein marker p_0 is present in the CNS of zebrafish from 72hpf. Similar expression has been found in other fish such as trout and shark and in early vertebrates such as *xenopus*. A co-expression of PLP and p_0 have been described in the same myelin cells in xenopus (Yoshida and Colman 1996). In zebrafish, most p_0 expressing cells co-localized with *plp* and also *mbp* as occurs in *xenopus*. The role of P_0 in the PNS of mammals is to compact myelin sheaths in Schwann cells -a similar function to PLP in the CNS. It is speculative that these two proteins have co-evolved to fulfil similar roles. Thus the absence of P_0 expression from the CNS myelin in terrestrial vertebrates suggests a

phylogenetic replacement of P_0 by PLP as the protein of choice for the CNS that has occurred relatively late in evolution. A possible mechanism for this change is the transposition of the fish P_0 promoter onto the DM20/PLP gene (Jeserich et al., 1997). However, there is no evidence, to date, to support this hypothesis.

Oligodendrocyte and myelin expression in the retina

Oligodendrocyte lineage and myelin specific genes were observed in cells in the GCL in close proximity to the nerve fibre layer, in a similar position to those described in the chicken retina (Fujita et al., 2000; Morcos and Chan-Ling, 2000; Nakazawa et al., 1993; Ono et al., 1998). Myelin basic protein and oligodendrocyte lineage markers *sox10*, *olig2* co-exist in the retina. This is in contrast with most mammals, except the rabbit. The retina of mammals are generally devoid of oligodendrocytes and thus myelin (ffrench-Constant, 1994).

In mammals, the lamina cribrosa at the head of the optic nerve stops the migrating OLPs from the optic nerve entering the retina in mouse. However as in chicken, this structure is absent in the zebrafish, presumably explaining why oligodendrocytes enter the retina.

Mutant *Otter* show defects in oligodendrocyte differentiation

The *otter* mutant showed a disrupted expression of *mbp* in the CNS and also PNS. Furthermore, I observed lack of myelin protein markers such as *mbp* and *plp* in the spinal cord although expression of the myelin protein marker p_0 persisted. Electron microscopy of the ventral region of the *otter* spinal cord showed the presence of glial processes. It is quite surprising to detect myelin sheath in the CNS of a mutant lacking myelin proteins such as *mbp* and *plp*. One possibility is that the loosely wrapped processes observed in the electron micrographs are due to the small amount of p_0 myelin marker detected. As this was very early in development, it is difficult to determine any difference compared to the wild type.

A similar situation has been found with a mouse mutant shiverer which has an autosomal recessive mutation caused by a deletion in the MBP gene (Roach et al., 1983; Molineaux et al., 1986). Shiverer mice do not produce MBP and have no compact CNS myelin (review see (Readhead and Hood, 1990). Behavioural analysis shows a generalized tremor, which

becomes progressively more prominent leading to convulsions and seizures around postnatal day 12 with early death. Morphological analysis of the CNS revealed an almost total lack of myelin. Myelin begins to form wraps but does not mature due to lack of MBP. In contrast, the PNS myelin of the shiverer mouse appears to be qualitatively normal, with normal thickness lamellar structure and periodicity. The absence of MBP does not prevent the formation of compact myelin in the PNS, being compensated by another PNS myelin protein, possibly P_0 (Martini et al., 1995). In *otter* mutant fish p_0 could possibly compensate for part of the absence of *mbp* and *plp* in the CNS. It would be interesting to study *otter* mutant fish at later stages when myelin in the wild type is fully compacted. However, due to the early mortality of this mutant this is not possible.

The mutated gene in *otter* mutant has not been identified to date. As the zebrafish genome project is completed it will be possible to determine a comprehensive map of the *otter* allele. Once the *otter* gene is identified, this mutant may be a valuable tool for the investigation of myelin structure and development and in particular the process of compacting myelin. The mammalian homologue of this mutant could also be cloned and studied.

Chapter 5

Regulation of Platelet Derived Growth Factor Receptor Alpha (PDGFRα) in Zebrafish OLPs

5.1 Introduction

It is well established that platelet-derived growth factor receptor alpha subunit (PDGFRα) identifies OLPs as soon as they are formed from a subset of neuroepithelial cells in the ventral half of the embryonic rat (E14), mouse (E12.5) and chicken (E7.5) spinal cord (Pringle et al., 1996; Pringle and Richardson, 1993). These PDGFRα positive cells disperse throughout the grey and white matter at later stages of development, persisting in mammals into adulthood (Chapter 1, Section 1.4.1.6). Yet, there has been no investigation of the expression of PDGFRα in relation to oligodendrocyte development in zebrafish or amphibia; although in other respects oligodendrocyte differentiation resembles that seen in mammals. This similarity in zebrafish has been described in Chapters 3 and 4 of this Thesis and (Park et al., 2002; Schweitzer et al., 2003). In *Xenopus* a number of studies using an oligodendrocyte specific antibody (olig) and myelin proteins markers (MBP and PLP) have shown that oligodendrocyte development also takes place in a characteristic ventral-to dorsal sequence, first appear in the ventral spinal cord and later spreading towards the lateral and dorsal white matter (Yoshida, 1997; Maier and Miller, 1995).

5.1.1 PDGFRα structure and expression

PDGF was initially isolated from blood platelets as a mitogen (growth factor) for connective tissue and glial cells (Kohler and Lipton, 1974; Heldin and Ostman, 1996). It is now known that PDGF and its receptors are expressed in many different cell types in embryonic development and in adult vertebrates (review by (Hoch and Soriano, 2003; Ataliotis and Mercola, 1997). Thus, PDGF plays a critical role during normal embryonic and postnatal development in kidney, lung and blood vessels, although overactivity is linked to different pathological processes which include tumor growth, angiogenesis, artherosclerosis, oncogenesis and wound healing (Hoch and Soriano, 2003; Heldin et al., 2002; Betsholtz et al., 2001; Ross et al., 1986; Ross et al., 1990).

PDGF is a dimeric molecule consisting of polypeptide chains A and B that form homo- and heterodimmers; PDGF-AA, PDGF-BB and PDGF-AB (Heldin and Westermark, 1999a; Heldin and Westermark, 1999b). Recently, two additional PDGF related peptides

have been identified PDGF-C and -D. These two poplypeptides form only homodimers PDGF-CC and PDGF-DD (Bergsten et al., 2001; LaRochelle et al., 2001; Li et al., 2000).

The PDGF dimers bind to and signal through two related protein tyrosine kinase receptors, the PDGF α– and β– receptors (PDGFRα and β). PDGF receptors have different ligand binding capacities; where PDGFRα binds PDGF-A, -B and-C while PDGFRβ binds PDGF-B and -D. The receptors form homodimers or heterodimers (αα, αβ,ββ) depending on the PDGF dimers available (Heldin and Westermark, 1999a).

Of the two receptors and four ligands of PDGF, PDGFRα and PDGF-A are the predominant forms present in the early embryo (Jones et al., 1993). Their expression pattern during early embryonic development in mouse and *Xenopus* are widespread, complex and mainly complimentary. Receptor and ligand are maternally co-expressed in all cells. From early gastrulation, PDGFRα and PDGF-A start to occupy an adjacent expression, PDGFRα expression is restricted to mesodermal cells, while PDGF-A is restricted to the adjacent ectodermal cells. After somatogenesis begins, PDGFRα is found expressed in mesodermal and neural crest derived tissues while PDGF-A is expressed in the adjacent ectoderm and endoderm (Orr-Urtreger and Lonai, 1992; Palmieri et al., 1992; Schatteman et al., 1992). A similar pattern has also been observed in *Xenopus* (Ataliotis et al., 1995; Jones et al., 1993). In addition, both ligand and receptor are also produced in neurons and glia precursors respectively in the mouse CNS, where most of the studies have been performed. In zebrafish, *pdgfrα* and *pdgf-a* have been cloned and characterised during embryogeneis by Liu et al (2002a,b). Ligand and receptor are maternaly co-expressed in all cells persisting until late gastrulation where *pdgfrα* is expressed in cranial and trunk neural crest cells and *pdgf-a* expression is restricted to anterior structures in zebrafish embryos (Liu et al., 2002a; Liu et al., 2002b). These authors also described a faded expression of both genes, disappearing completely at later stages.

PDGFRα identifies OLPs in the ventral VZ of the E13 mouse spinal cord and ventral regions in the forebrain spreading thoughout the whole spinal cord and areas of the brain (Pringle et al., 1991; Pringle and Richardson, 1993; Tekki-Kessaris et al., 2001; Woodruff et al., 2001; Yeh et al., 1993). In the CNS, PDGF-A mRNA is first detected in the floor

plate in the E11.5 mouse embryo. After E13, when PDGFRα OLP positive cells are being produced, PDGF-A transcript can be detected in neurons in all parts of the spinal cord and the brain (Yeh et al., 1993; Calver et al., 1998b; Hutchins and Jefferson, 1992; Orr-Urtreger et al., 1992). PDGFRα and PDGF-A expression persists throughout life in OLPs and neurons respectively (Yeh et al., 1993).

In vivo, a dramatically reduced number of PDGFRα-positive OLPs and myelinating oligodendrocytes is found in the embryonic brain of PDGF-A deficient mice (Calver et al., 1998). Hence, PDGF-A can regulate OLP proliferation (Calver et al., 1998; Fruttiger et al., 1999; van Heyningen et al., 2001; Durand and Raff, 2000). PDGF-A mutants that survive postnatally become severely hypo-myelinated, developing a tremor phenotpye similar to mutants with defects in myelin (Fruttiger et al., 1999). PDGFRα knockouts show a severe phenotype, and die between E8 and E16. Typically, defects include cleft face, spina bifida, skeletal and vascular defects (in the axial skeleton and neural crest derived mesenchyme, specifically in the cardiac outflow tract and the head (Soriano, 1997). *In vitro* experiments have shown that PDGFRα function is required for the proliferation, survival and differentitation of OLPs (Hall et al., 1996). In *Xenopus* and zebrafish, a defect of the ligand and receptor is also lethal but no specific effects on oligodendrocyte development have been reported (Liu et al., 2002a; Liu et al., 2002b).

5.1.2 Transcriptional regulation of the PDGFRα gene expression

The human *PDGFRα* gene is about 64 kb and consists of a total of 23 exons (Figure 5.1A). PDGFRα genes for human, rat and mouse are 90-95% homologous at the amino acid level and share 73% homology with *Xenopus* and 74 % with zebrafish (Liu et al 2002a). The PDGFRα gene is located on human chromosome 4, mouse chromosome 5 and in the zebrafish is mapped to linkage group (LG)-20. PDGFRα in human, mouse or zebrafish are located close to the c-KIT gene, a member of the platelet derived growth factor family of tyrosine kinase receptors (Mol et al., 2003; Hsieh et al., 1991; Spritz et al., 1994).

PDGFRα is strictly regulated during embryogenesis (Mercola et al., 1990; Palmieri et al., 1992; Rappolee et al., 1988; Orr-Urtreger and Lonai, 1992; Schatteman et al., 1992). The regulatory mechanisms that control the time and tissue specific expression of PDGFRα are at present unkown. From a number of studies, PDGFRα expression in different tissues or cell types appears to require specific regulatory elements and different transcription factors. A 2.2 kb human *PDGFRα* upstream sequence cloned in front of a *lacZ* reporter gene correctly drives the β–galactosidase (β-gal) expression in mesoderm and neural crest derived tissues of the developing mouse, compared to the distribution of PDGFRα mRNA expressed from the endogenous mouse gene (Zhang et al., 1998). This fragment, however, did not appear to drive PDGFRα expression in OLPs. Groups investigating PDGFRα regulation, particularly in OLPs, have shown that PDGFRα transcribes from the same transcriptional start site in OLPs and also other cell types, but distant *cis*-acting enhancer elements are required for PDGFRα expression in OLPs (Sun et al., 2000); Flores-Garcia, UCL Thesis). A yeast artificial chromosome (YAC) vector containing the human PDGFRα gene flanked by 3.5 kb of upstream sequence and 300 kb of downstream (380kb YAC) sequence has been expressed in spinal cord OLPs (Lu et al., 2002). A similar pattern of expression was also seen with a bacterial artifical chromosome (BAC) transgene containing the human *PDGFRα* gene with 3.5 kb of upstream sequence and 47kb of downstream sequence (Flores-Garcia, UCL Thesis).

This chapter describes the expression of *pdgfrα* in zebrafish and *Xenopus* spinal cord in comparison to the glial cell specific marker, *sox10* from early development until adult stages. Zebrafish *pdgf-a* expression pattern is also described. In addition, I perform a preliminary transgenic analysis to determine whether the long-range regulatory elements that direct *PDGFRα* expression in the OLPs of higher vertebrates can function in zebrafish.

This could provide some insight into the evolution of gene regulation in general and the evolution of oligodendrocytes in particular.

5.2 Results

5.2.1 *Pdgfrα* and *pdgf-a* expression in zebrafish

To examine the temporal and spatial expression of *pdgfrα* and *pdgf-a* during zebrafish embryonic development I performed *in situ* hybridizations on whole mount preparations and sections using DIG labelled antisense RNA probes. In parallel, sense probes (homologous to mRNAs) were used as controls for the specificity of the reaction. At 24hpf strong *pdgfrα* staining was present along the head and the trunk (Figure 4A, C, E). The highest levels of zebrafish *pdgfrα* mRNA was found in the most anterior regions including the eye lens. Along the trunk the expression was confined to scattered cells located dorsally and ventrally showing characteristics similar to those of the sclerotome and trunk neural crest population (Figure 4) as *colourless* and Ret1 showed similar distributions at this stage (Dutton et al., 2001a; Marcos-Gutierrez et al., 1997). A cross-section of the trunk shows the precise localization of *pdgfrα* expression in cells on the dorsal, ventral and lateral side of the spinal cord. This expression is conserved in both mouse and *Xenopus*.

The highest level of zebrafish *pdgf-a* expressing cells was at 24hpf development and was found in more anterior regions, towards the head. No expression was seen in the eye lens. The *pdgf-a* mRNA cells in the trunk were restricted to a band of cells located dorsally. From a dorsal view, these *pdgfrα* and *pdgf-a* mRNA-expressing cells are localized bilaterally (Figure 5.1D, H). *Pdgfrα* expression was also present in the retina at later stages (Figure 5.1J).

Pdgf-a mRNA *in situ* hybridization analysis in transverse spinal cord sections through the trunk of 72hpf zebrafish embryo showed very similar expression in the dorsal regions to that observed at 24hpf. A small number of *pdgf-a* expressing cells are located in the ventral regions of the spinal cord. From their close central position in the spinal cord these cells appear to be neurons, however, no further characterization has been done. At later stages

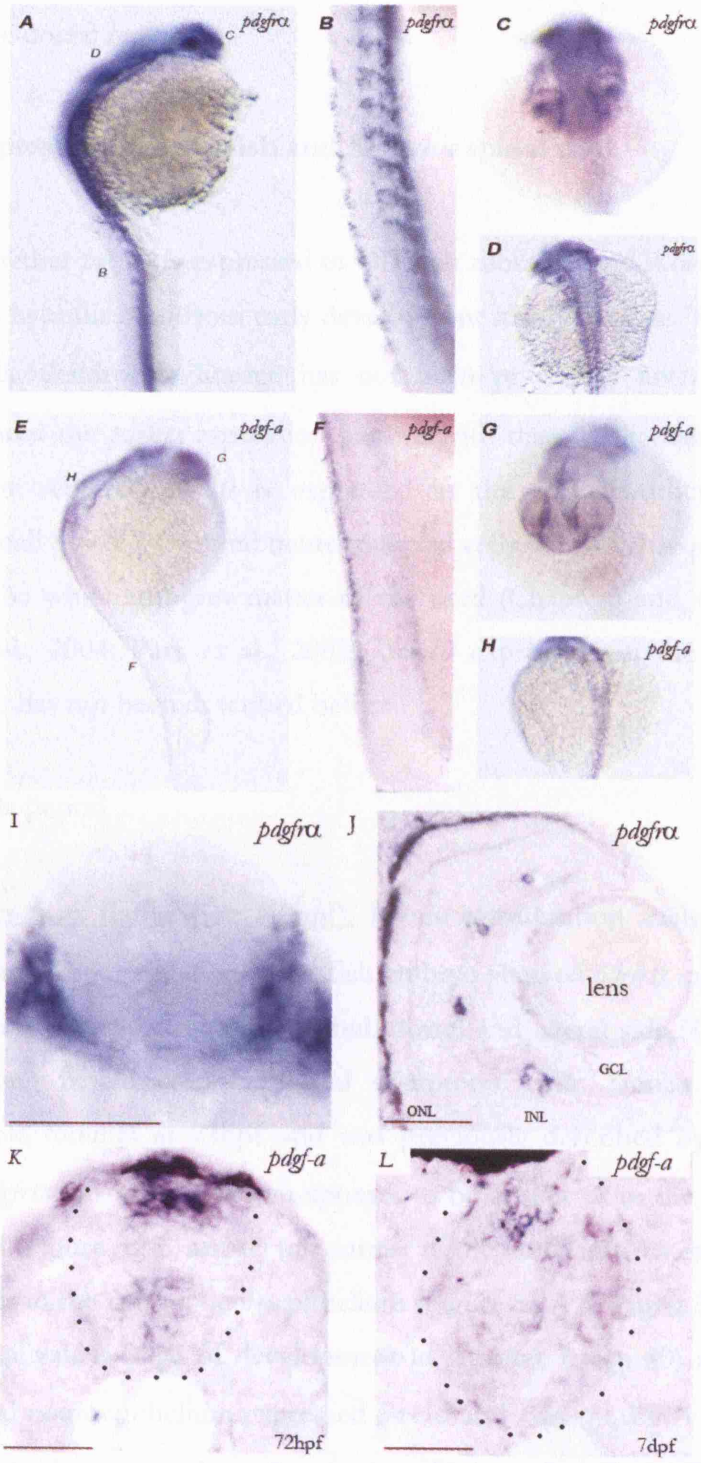


Figure 5 1. Zebrafish *pdgfrα* and *pdgf-a* mRNA expression. Whole mouted 24hpf showng high levels of mRNA mainly in anterior regions but also in the spinal cord (**A-H**). *Pdgfrα* expression in a cross section of whole mounted embryoat 24hpf (**J**). *Pdgfrα* expression in the retina.(**I**). *Pdgf-a* expression at 72hpf and 7dpf localized mainly in dorsal region of spinal cord (**K-L**). *ONL*, Outer nuclear layer; *INL*, Inner nuclear layer; *GCL* Ganglion cell layer. Scale bars: 20μm

(7dpf), *pdgf-a* expression in the spinal cord occur is weaker but a small number of cells can be detected in the dorsal region.

5.2.2 *Pdgfrα* expression in zebrafish and *Xenopus* spinal cord

To determine whether *pdgfrα* is expressed in OLPs in zebrafish and *Xenopus* spinal cord, I performed *in situ* hybridization from early development to adult stages. The expression of *pdgfrα* in the oligodendrocyte lineage has not been previously investigated in either species. I compared the *pdgfrα* expression pattern with that of oligodendrocyte specific marker *sox10*. In zebrafish *sox10* is expressed in the oligodendrocyte lineage, first appearing in a small group of ventral neuroepithelial cells which subsequently proliferate and migrate to the white and grey matter of the cord (Chapter3 and 4; (Dutton et al., 2001b; Park et al., 2004; Park et al., 2002). *Sox10* expression in the oligodendrocyte lineage in *Xenopus* has not been described before.

5.2.2.1 Pharyngula period

Forty eight hours post fertilization (48hpf). I*n situ* hybridization analysis in transverse spinal cord sections through a 48hpf zebrafish embryo showed *pdgfrα* mRNA expression in cells surrounding the spinal cord at ventral, dorsal and lateral side. These most likely represent migrating neural crest cells and sclerotome cells. Similar expression was observed in whole mounts at 24hpf and was previously described by Liu et al 2001 (Figure 5.2C). *Pdgfrα* mRNA expression appears to be absent from the neuroepithelium of the spinal cord (Figure 5.2C, arrow) in contrast to the *sox10* mRNA expression located in a cluster of cells in the ventral neuroepithelium (Figure 5.2A, Chapter 3). In spinal cord sections at the equivalent stage of development in *Xenopus,* (stage 40) a small group of cells in the ventral neuroepithelium expressed *Sox10* and *Pdgfrα* mRNA (Figure 5.2B,D). Double *in situ* hybridization with probes against *Xenopus Sox10* and *Pdgfrα* showed an overlap of both transcripts (data not shown).

5.2.2.2 Hatching period

Seventy-two hours post-fertilization (72hpf). By this stage zebrafish *sox10* mRNA is expressed in single cells away from the neuroepithelium and throughout the entire spinal cord including grey matter and white matter (Figure 5.2E). When I analysed *pdgfrα* at this stage it continued to be expressed in tissues outside the CNS including a subset of cells around the spinal cord, but not in the CNS itself (Figure 5.2G). This is in disagreement with Liu et al (2002a) who describe a complete dissapearance of *pdgfrα* expression by this stage of development. *Sox10* (Figure 5.2F) and *Pdgfrα* (Figure 5.2H) mRNA expression at the equivalent stage of development in *Xenopus* (stage 42) was localized in individual cells dispersed throughout the grey and white matter of the spinal cord.

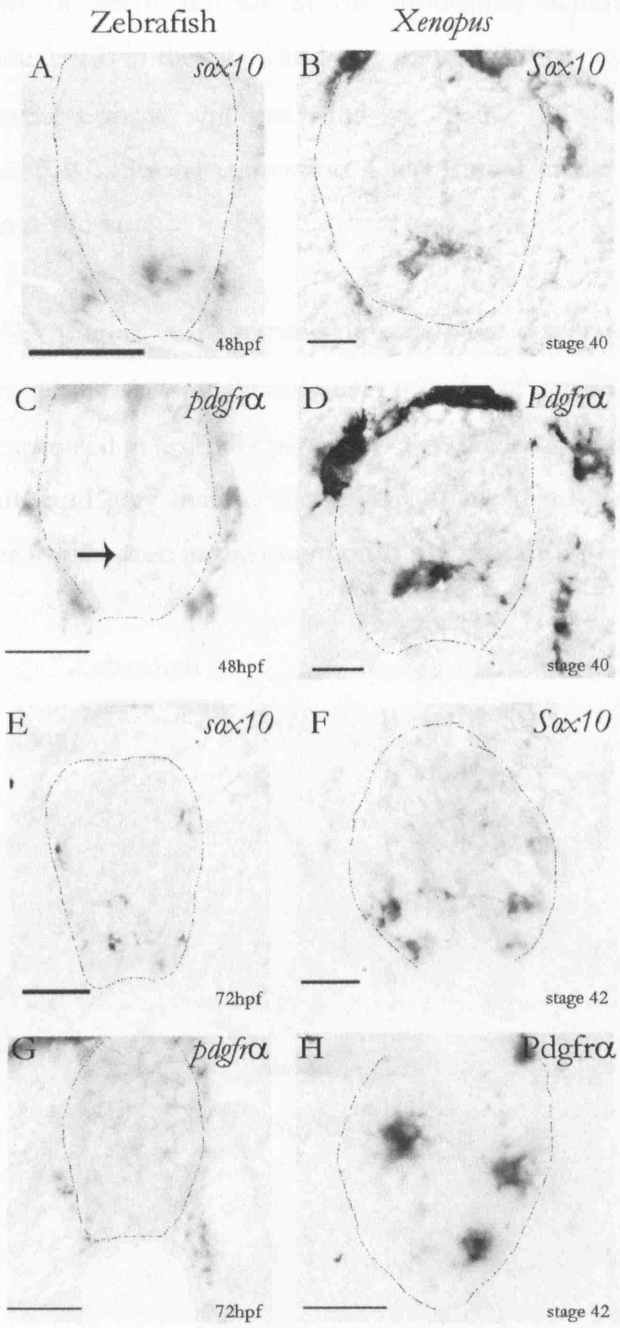


Figure 5.2 *Pdgfrα* and *Sox10* expression in zebrafish and *Xenopus* spinal cord. 48hpf and 72hpf (**A,B,E,G**) in zebrafish and comparable stages, stage 40 and 42 (**C,D,G,H**) in *Xenopus*. At 48hpf in zebrafish and *Xenopus* stage 40, in, neuroepithelial expression of *sox10* is localized in the ventral neuroepithelium close to the floor plate, these cells proliferate and migrate throughout the spinal cord. *Pdgfrα* mRNA expression in *Xenopus* spinal cord resembles the expression of *Sox10*. Zebrafish *pdgfrα* mRNA expression was localized in the outside periphery of the spinal cord, and completely absence in the CNS. Scale bars: 20 μm

In situ hybridization of *sox10* mRNA in one-month-old zebrafish showed mRNA expression in individual cells in the white and grey matter of the spinal cord (Figure 5.3A). Zebrafish *pdgfrα* expression is still restricted to tissues outside the CNS e.g the mesenchyme (Figure 5.3C). Strong expression is also noted in the retina of one-month old zebrafish (data not shown).

Xenopus Sox10 mRNA (Figure 5.3B) expressing cells were dispersed uniformly in white and grey matter throughout the spinal cord at six months of age. *Xenopus Pdgfrα* mRNA (Figure 5.3D) was expressed in a similar pattern to *Sox10* with cells distributed all through the spinal cord white and grey matter. Expression of *sox10* mRNA in a six-month-old zebrafish was similar to that seen in the one-month-old zebrafish (data not shown).

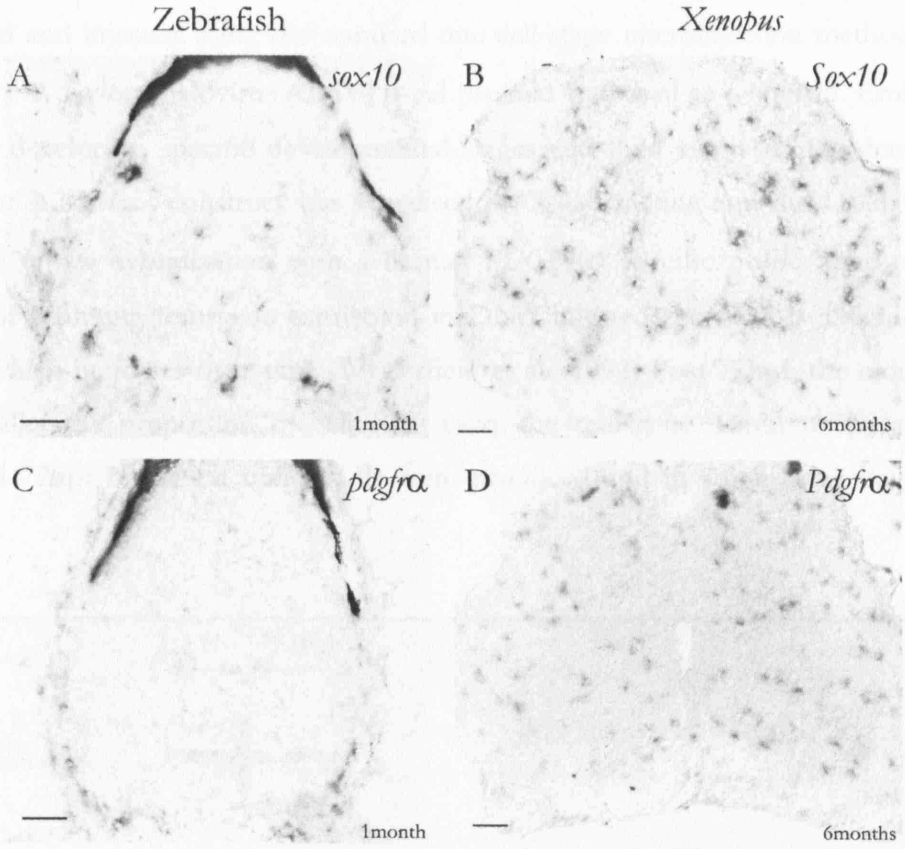


Figure 5.3 Pdgfrα and Sox10 expression in adult zebrafish and *Xenopus* spinal cord. Transverse sections through one-month-old zebrafish and six-month-old *Xenopus* spinal cord showing the expression of *sox10* (**A-B**) and *pdgfrα* (**C-D**). *Sox10* positve cells in adults proliferate and expand covering the white and grey matter of the spinal cord in both species (**A-B**). *Pdgfrα* is expressed in cells outside the spinal cord in zebrafish (**C**) and in dispersed single cells throughout the white and grey matter in *Xenopus* (**D**). Scale bars: 20μm

5.2.3 Human *PDGFRα* expression in zebrafish spinal cord

A transient transgenic expression approach was used to investigate whether human *PDGFRα* regulatory elements previously characterised in transgenic mice (Zhang et al., 1998; Sun et al., 2000); Flores-Garcia., UCL Thesis) could drive cell type specific expression in zebrafish OLPs.

5.2.3.1 Injection of the human PDGFRα 2.2kb-*lac Z* and 110kb BAC constructs in one cell stage zebrafish

The 2.2 kb upstream, human *PDGFRα* promoter-*lacZ* construct (2.2kb-*lacZ*) (Afink 1995) and human 110kb *PDGFRα* BAC construct (110kb BAC) (Flores-Garcia, UCL Thesis) were purified and injected using the standard one cell stage microinjection method (see Methods 2.7). A Cytomegalovirus (CMV) β-gal plasmid was used as a control. Embryos were left to develop to specific developmental stages and then analysed. The transient expression of 2.2kb-*lacZ* construct was visualised by X-gal staining and the 110kb BAC construct by *in situ* hybridization with a human *PDGFRα* specific probe. One of the drawbacks of analysing transgene expression in DNA injected zebrafish is a significant mosaicsm, which increases over time (Westerfield et al., 1992) Post 72hpf, the older the fish the smaller the proportion of cells that carry the transgene. Embryos were only studied until 72hpf by which time OLPs were produced and in some cases began to differentiate.

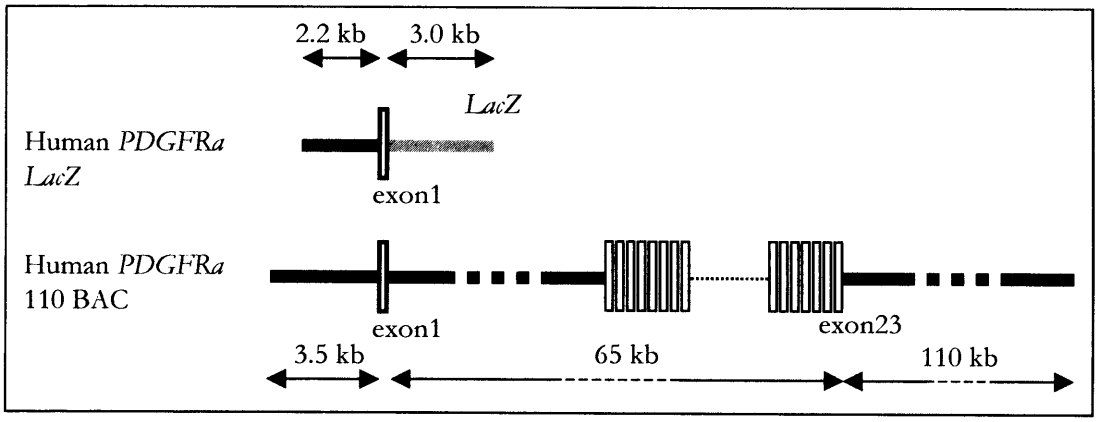


Figure 5.4 Human *PDGFRα* constructs used in microinjection. Diagrams of the **A.** genomic human *PDGFRα* 2.2kb-*lacZ*. **B** genomic human *PDGFRα* 110kb BAC

5.2.3.2 Transgenic analysis of human *PDGFRα* 2.2kb-*lacZ* construct in zebrafish spinal cord

At 19hpf, whole mounted embryos injected with 2.2kb-*lacZ* construct showed a mosaic β-gal activity predominantly in notochord and mesenchymal cells bordering the spinal cord (Figure 5.6 A). Sample trunk cross-sections showed the *lacZ* staining distributed in cells of the notochord and surroundings of the spinal cord (Figure 5.6A,C,D). At the pharyngula stage of 48hpf, whole mounted embryos showed a reduction in the frequency of β-gal positive cells but their distribution remained similar (Figure 5.6B,E,F). β–gal expression was detected only at very low levels in the notochord at this stage of development. Approximately 400 embryos were analysed with similar results showing transgene expression pattern that was grossly similar here to that of the endogenous *pdgfrα* in tissues of mesenchymal and neural crest origin (Figure 5.2) and to the expression in the transgene mouse (Figure 5.5). Zebrafish control embryos injected with CMV-*lacZ* plasmid showed β-gal positive cells distributed randomly throughout the embryo including the CNS (data not shown).

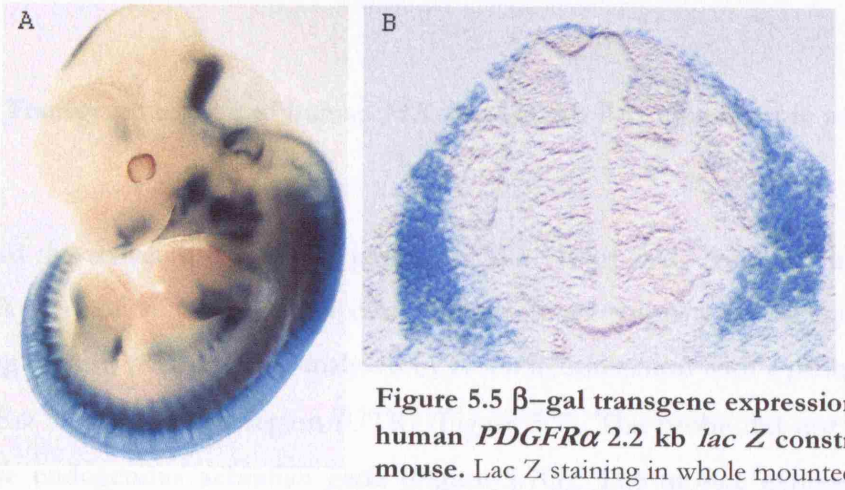


Figure 5.5 β–gal transgene expression driven by human *PDGFRα* 2.2 kb *lac Z* construct in E10 mouse. Lac Z staining in whole mounted (**A**) and its corss sectioned (**B**) from transgenic mice showing expression in the mesenchyme around spinal cord. Data from Flores Garcia, UCL Thesis.

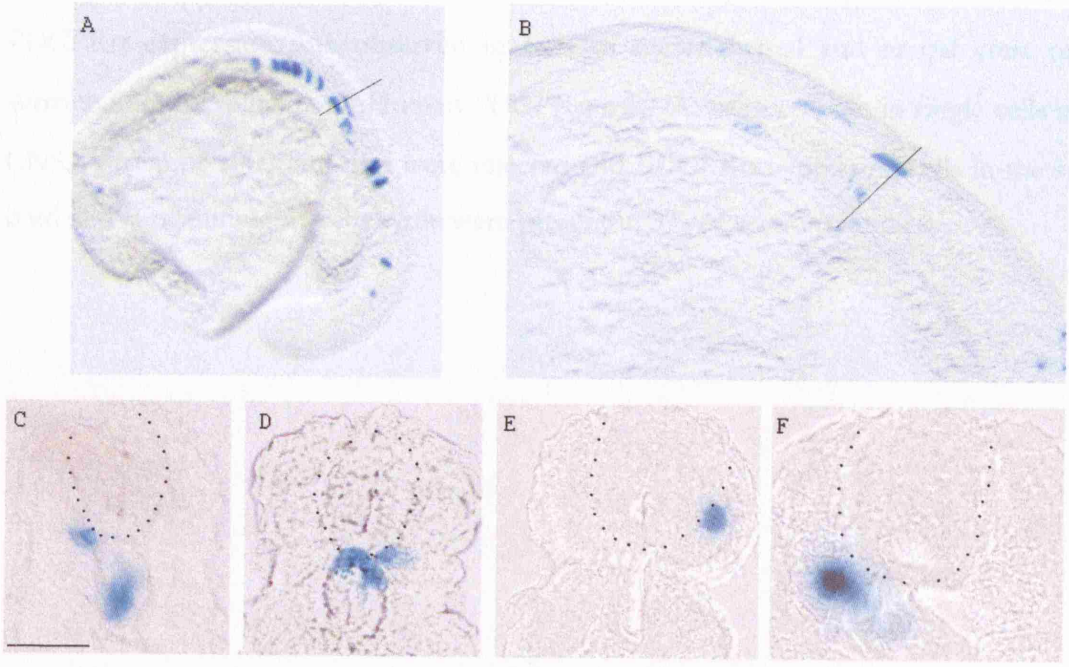


Figure 5.6 Mosaic βgal transgene expression driven by human *PDGFRα* 2.2kb *lacZ* construct in zebrafish. Whole mounted zebrafish embryos and cross sections across the trunk illustrate β–gal transient transgene expression in notochord and cells around the spinal cord at 19hpf (**A,C,D**) and 48hpf (**B,E,F**). Zebrafish whole mounted embryos are orientated anterior to the left and dorsal up. Scale bars: 20μm

5.2.3.3 Transgenic analysis of human *PDGFRα* 110kb BAC construct in zebrafish spinal cord

I studied the transient expression pattern of the 110kb BAC construct injected into the one-cell stage of zebrafish spinal cord. Trunk cross-sections at pharyngula (48hpf) and hatching (72hpf) periods were analysed by *in situ* hybridization with a probe to the human *PDGFRα* 3' untranslated region (UTR) (Figure 5.7). The probe did not cross-hybridize with the endogenous zebrafish gene (Figure 5.7G). The mosaic expression pattern of human *PDGFRα* transcript was studied in injected fish and compared with the conserved endogenous *pdgfrα* mRNA expression in mesenchyme and neural crest cells (Figure 5.6A). Individual human-*PDGFRα* positive cells were observed in single cells in the ventral spinal cord from 48hpf (Figure 5.7B, C, E, F, H). Although, the number of cells stained

per section varied, a similar expression pattern was found at 48hpf and 72hpf of development. As with the 2.2kb-*lacZ* construct, the expression pattern of the human *PDGFRα* expression was observed in cells of mesenchymal and neural crest origin, surrounding the spinal cord. Human *PDGFRα* mRNA was also seen in single cells in the CNS. A total of 1000 embryos were injected and *PDGFRα* expressing cells in the spinal cord and surrounding mesenchyme were present in 3% of sections studied.

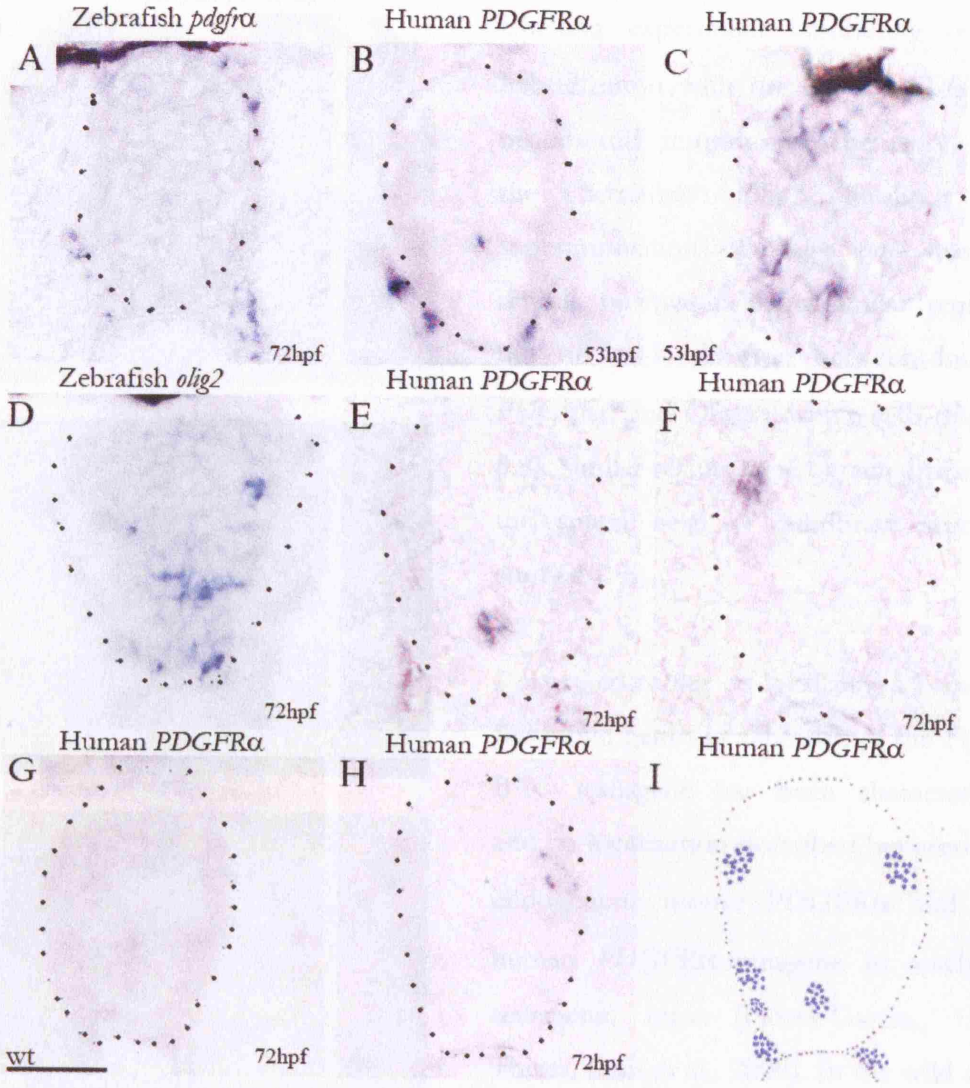


Figure 5.7 Human *PDGFRα* 110kb BAC transient transgene expression in spinal cord of injected zebrafish. Zebrafish *pdgfrα* endogenous expression is localized in cells surrounding the spinal cord (**A**). Mosaic transgene expression in zebrafish spinal cord showed human *PDGFRα* mRNA expression localized in cells in the spinal cord mainly in the white matter (**C,F,H**). There is some overlapping expression between endogenous *pdgfrα* and human *PDGFRα* in cells outside the spinal cord (**B,E**). Expression summary map collected from embryos injected with 110kb BAC. Dots indicate the human *PDGFRα* mRNA expression (**I**). Oligodendrocyte lineage marker *olig2* in zebrafish spinal cord is observed in cells in the neuroepithelium and throughout the white matter (**D**). Human *PDGFRα* probe in wild-type embryo spinal cord (**G**). Scale bars: 20μm.

To determine whether the human *PDGFRα* positive cells localized in the spinal cord of injected embryos were OLPs, I performed labelling experiment combining *in situ* hybridization with the human *PDGFRα* probe and immunohistochemistry with the zebrafish Olig2 antibody. A superimposition of these two markers reveals positive cells in similar regions, but no co-localization between human *PDGFRα* and Olig2 positive cells (Figure 5.8). Similar results were obtained from all the spinal cord or hindbrain sections studied (>6).

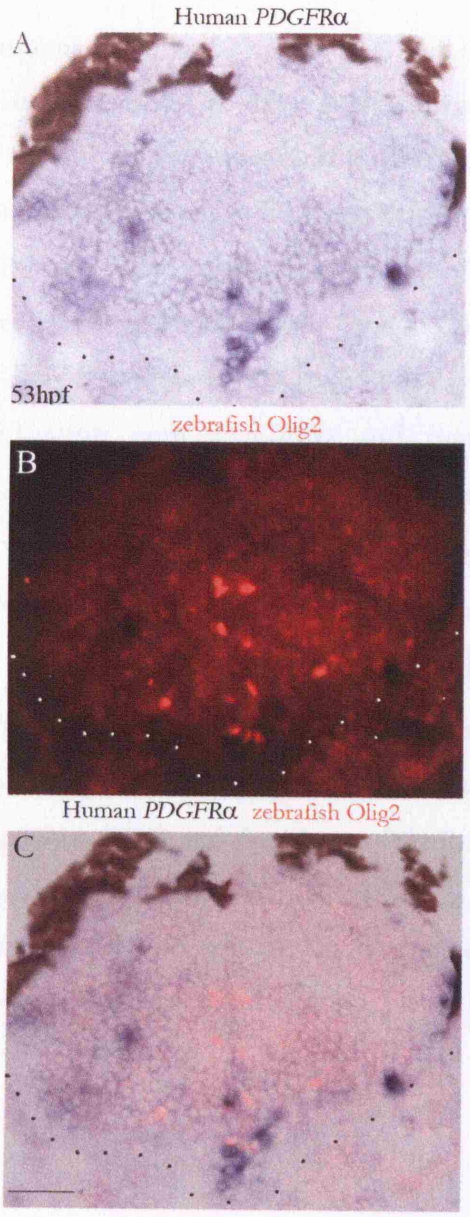


Figure 5.8 Double labelling for human *PDGFRα* and zebrafish Olig2 protein. Human *PDGFRα* mRNA by *in situ* hybridization (blue) (**A**) and zebrafish Olig2 antibody (red, IMF) (**B**) in injected zebrafish at 53hpf of development. There is no overlap between human *PDGFRα* and Olig2 (**C**). Scale bars: 20μm

I expected to see co-localization between these two genes in zebrafish as the 110kb BAC transgene has been characterised and co-localization described between the endogenous mouse PDGFRα and the human *PDGFRα* transgene in newborn transgenic mice (Flores-Garcia., UCL Thesis, (Sun et al., 2000). In the wild type mouse, oligodendrocyte lineage marker Olig2 and OLP marker *PDGFRα* co-localized in OLPs from early stages of development. By analogy I expected human *PDGFRα* to co-localize with zebrafish Olig2. As I had analysed transient transgene expression at stage 48-72hpf in zebrafish, (which is equivalent to E13.5-E15 in mouse). I decided to study whether there was co-localization of human *PDGFRα* and mouse Olig2 in the transgenic mouse at embryonic, as apposed to postnatal stages.

5.2.3.4 Human *PDGFRα* upregulation in OLPs in mouse spinal cord

Transgenic mice with 380kb YAC and 110kb BAC human *PDGFRα* contain the *cis*-regulatory elements to drive expression in OLPs. Spinal cord sections of these mice at E13 display ectopic expression in the dorsal spinal cord. Ventral expression appeared to be in the same region to OLPs production (Sun et al., 2000), Flores-Garcia, UCL Thesis). By P0 and later stages the human *PDGFRα* expression completely simulates the expression of the endogenous gene in OLPs. Sections from these transgenic embryos at early E13.5 and later P4 stages of development were subjected to combine *in situ* hybridization with *PDGFRα* and immmunostaining with mouse oligodendrocyte antibody Olig2. At P4 most, if not all, human *PDGFRα* positive cells co-localized with Olig2 positive cells (Figure 5.9). However, at E13.5 hardly any human *PDGFRα* mRNA positive cells co-localized with immunostained Olig2 cells (Figure 5.10). This indicates that the 110kb or 300kb human *PDGFRα* genomic clones do not contain all the regulatory elements for *PDGFRα* expression in early developing OLPs. This could explain why at early stages of zebrafish development there is no co-localization between endogenous Olig2 and the human *PGFRα* transgene in injected embryos. Unfortunately, studying co-localization at later stages has not been practical by transient transgenesis, as few cells retain and express the transgene DNA for long periods of time. To examine later stages it will be necessary to generate stable germline transgenic fish.

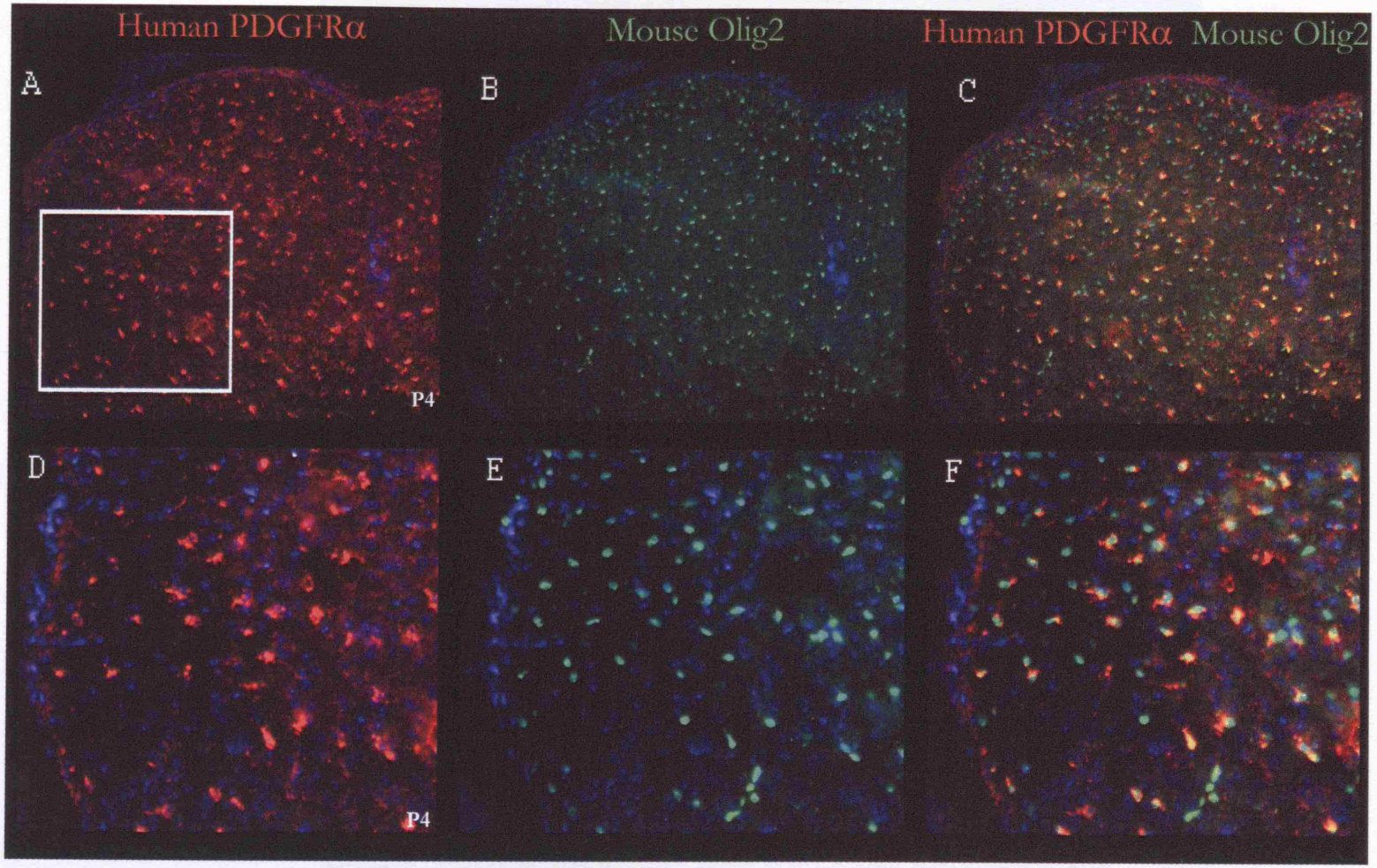


Figure 5.9 Human PDGFRα upregulation in OLPs in P4 mouse spinal cord. Double labelling of human PDGFRα mRNA (red,ISH) (**A**) and mouse OLIG2 protein (green,IMF) (**B**) in 110kb BAC transgenic mice at postnatal day four (P4). Most of the human PDGFRα expressing cells overlap with mouse Olig2 positve cells (**C**).Higher-magnification images of the areas delineated in white (**D-F**). Sections were counterstained with DAPI to localize all nuclei.

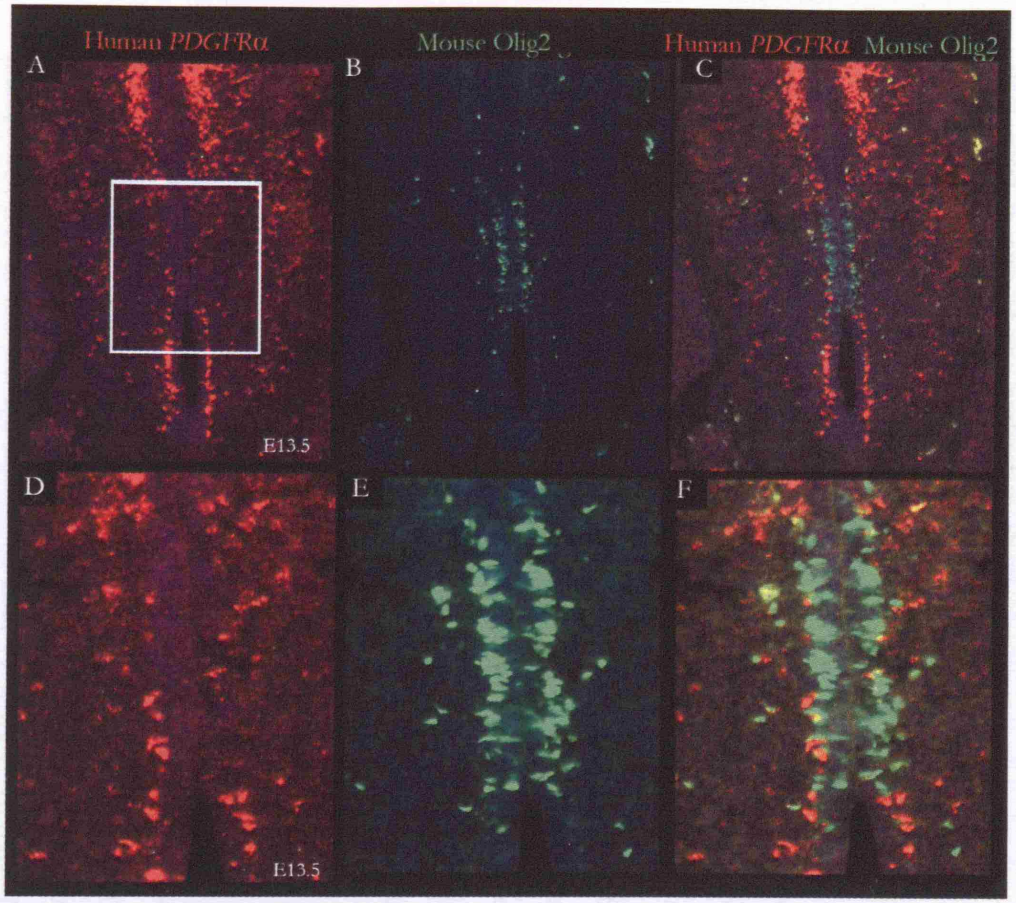


Figure 5.10 Human *PDGFRα* upregulation in OLPs in E13.5 mouse spinal cord. Double labelling of human *PDGFRα* mRNA (red, ISH) (**A**) and mouse Olig2 protein (green,IMF) (**B**) in 380kb YAC transgenic mouse at E13.5. There is no overlap between human *PDGFRα* expressing cells with mouse Olig2 positive cells (**C**) Higer magnification images of the area delineated in white (**D-F**)

5.3 Discussion

Absence of expression of *pdgfrα* in zebrafish CNS

PDGFRα is one of the earliest markers identifying oligodendrocyte progenitors in the ventral half of the embryonic mouse, rat and chicken (Pringle et al., 1996; Pringle and Richardson, 1993). In *Xenopus*, as reported here, *Pdgfrα* mRNA expression follows a very similar pattern to that of higher vertebrates. Expression starts in a few cells in the ventral region (stage 40) of the spinal cord, proliferating and migrating throughout the white and grey matter until more mature stages. Our results and those reported by Liu et al., (2002) suggest that the expression pattern of *pdgfrα* in zebrafish CNS is different to that in mouse, chicken and *Xenopus*. Surprisingly, it appears not to be expressed in the CNS of the zebrafish at any of the stages studied. However, *pdgfrα* expression continues at low levels in tissues outside the CNS such as retina, neural crest and mesenchymal cells in the trunk of the zebrafish, at least until juvenile period. It does not disappear completely by 72hpf, as suggested by Liu et al (2002). Furthermore, *pdgfrα* expression continues in other tissues outside the CNS.

The reason why *pdgfrα* is absent in zebrafish CNS is not known. A second copy of the gene might be expressed in CNS following the fish specific genome duplication (Amores et al., 1998; Taylor et al., 2003) review (Meyer and Van de, 2003). It is thought that during the evolution of vertebrates, a duplication of the entire genome occurred in the fish lineage but not in the lineage leading to land vertebrates (tetrapods) as two paralogouos copies of a large number of gene families were found in fish and only one ortholog in tetrapods (Loosli et al., 1998). Such a *pdgfrα* gene duplication event in zebrafish is unlikely since, following a search of the databases (NCBI and Ensemble), no other *pdgfrα* gene was found. Though the zebrafish genome is not completed yet, no duplicate gene exists in the database for another teleost fish, *Fugu rubripes*.

Regulation of PDGFRα

The expression of PDGFRα or indeed any gene, requires precise *cis*-regulatory elements in the DNA surrounding the gene and *trans*-acting transcription factors that control it by direct protein-DNA and protein-protein interaction. Changes in the expression of a particular gene could result from alterations either in the sequence of its *cis*-regulatory elements or the concentration or spatial distribution of *trans*-acting transcription factors, or both. Since a transcription factor usually interacts with many promoters (*cis*-regulatory sequences), it is more probable that the change would be in the regulatory sequences than in transcription factors (Tautz, 2000). The specific *cis*-regulatory element(s) required for PDGFRα expression in OLPs is still unknown. However, the region flanking the human *PDGFRα* gene which is required for the expression in OLPs in newborn transgenic mice has been narrowed down to a region 47kb downstream of exon 23. Within this region there is a single ~200bp element that is conserved among human rodent and chicken, but that is absent in zebrafish or *Fugu rubripes* (Sun et al., 2000); Flores-Garcia, UCL thesis). The absence of *pdgfrα* expression in zebrafish CNS (OLPs) could be due to a lack of OLP specific *cis*-regulatory elements in this fragment. However, if the *trans*-acting transcription factors that upregulate the *cis*-regulatory elements are conserved in zebrafish, then human *PDGFRα* regulatory elements should still be functional in fish and be upregulated in OLPs.

Here, using a transient transgenic expression in zebrafish, I have shown that human *PDGFRα 2.2*kb-*lacZ* fragment (2.2kb-*lacZ*) can establish the correct spatio temporal expression in mesoderm and neural crest derived tissues, similar to the endogenous expression in zebrafish. This suggests that it drives expression in similar tissues to those targeted in transgenic mouse (Zhang et al., 1998). The 380kb YAC and 110kb BAC that contain the small fragment 2.2kb upstream in their constructs also drive human transgene expression in these tissues in mouse as well as in zebrafish (Figure 5.6; Figure 5.8; (Sun et al., 2000); Flores–Garcia UCL thesis). Thus, the specific PDGRα regulatory elements that drive expression in mesoderm and neural crest derived tissues appear to be conserved between zebrafish and mammals. A number of other studies have shown that of the many genes that have a conservative expression, some of their *cis*-regulatory sequences

can be conserved for time periods regulating gene expression across species. For example, specific *cis*-regulatory regions in upstream and downstream positions have retained their regulatory function influencing the expression of the Shh gene across species in the ventral neural tube and notochord (Muller et al., 2000), as well as the expression of the Pax6 gene in lens, retina, pancreas, parts of neural tube and forebrain (Griffin et al., 2002; Kammandel et al., 1999; Kleinjan et al., 2004; Xu et al., 1999). There are also a number of zebrafish transgenic lines that have been created using heterologous mammalian promoters that recapitulate the endogenous gene expression (Yoshida and Macklin, 2005; Udvadia and Linney, 2003).

The 380kb YAC and 110kb BAC also drive human *PDGFRα* transgene expression in OLPs in newborn transgenic mice. This has been shown by double labelling experiments where most, if not all, the human *PDGFRα* postive cells overlap with the endogenous mouse PDGFRα or the oligodendrocyte antigen OLIG2 (Flores-Garcia, UCL Thesis, Figure 5.8). At E13.5 in transgenic mice, the expression of human *PDGFRα* transgene is localized in ventral regions of the neuroepithelium reminescent of oligodendrocyte production, although there is also ectopic expression of the transgene in dorsal regions of the spinal cord (Sun et al., 2000), Flores-Garcia, UCL Thesis, Figure 5.9). Double labelling with human *PDGFRα in situ* and Olig2 antibody showed, however, there is not that co-localization at this stage between OLPs and *PDGFRα* transgene expression, so the ventral as well as the dorsal expression appears to be ectopic (Figure 5.8). This dorsal and ventral ectopic expression progressively disappears by E15.5 and the transgene is expressed faithfully in OLPs.

Here, 110kb BAC transient transgene expression was observed in single cells in the zebrafish CNS unlike the endogenous expression, but similar to the oligodendrocyte expression pattern of *olig2* and *sox10* markers in cells throughout the spinal cord, as described in Chapter 3. Double labelling experiments were performed to check whether these cells were OLPs. However, human *PDGFRα* transgene expression did not co-localize with oligodendrocyte markers (Olig2) in zebrafish CNS (Figure 5.7).

It is possible that human *PDGFRα* positive cells in the spinal cord of transient transgenic zebrafish reflect the same activity of the *cis*-regultory elements that drive the early ectopic

expression in the E13.5 trasngenic mice. In general, the experiments from this Chapter shows that human *PDGFRα* transgene expression from a 110kb BAC construct injected in zebrafish resemble the expression of the transgenic mice at early stages.

To investigate whether the human OLP regulatory elements function properly during later development in fish would require production of a permanent transgenic line. This would overcome the problem with mosaicsm in older injected fish and allow the expression pattern to be analysed at later stages.

It is probable that all fish lack OLP specific regulatory elements and that new *cis*-regulatory elements or binding sites in the PDGFRα DNA have been acquired later in evolution. A similar example of a novel gene function being acquiered in tetrapods occurs with proteolipid protein (PLP), the main myelin protein in the CNS and its isoform DM-20. DM-20 is undetectable in vertebrates, such as amphibia and fish, but detectable in reptiles, birds and mammals (Waehneldt et al., 1985). It has been proposed that the DM-20 form was generated later in evolution by the introduction of a single base change or alternative splice site into the protein coding region in the Plp gene (Karthigasan et al., 1991; Yoshida and Colman, 1996).

A second possibility involves modifications of the pre-existing *cis*-regulatory elements of the PDGFRα OLP gene. An example comes from how insects (e.g. *Drosophila*) have lost abdominal limbs, whilst crustaceans have abdominal and thoracical limbs (Galant and Carroll, 2002). Both groups provide evidence that functional changes in a protein called Ultrabithorax (Ubx), which is encoded by a homeodomain gene, cause the suppression of *Distalless* (*Dll*) expression and abdominal limbs in insects. *Dll* gene is required for limb formation, Ubx protein repress its expression in the anterior abdomen of the *Drosophila* embryo. However, in crustaceans all of the developing limbs contain high levels of Ubx without altering the expression of *Dll* in the same tissue (Ronshaugen et al., 2002; Galant and Carroll, 2002). We might expect to find similar acquisition of new binding sites for transcription factors in the zebrafish *pdgfrα* gene.

Another possibility is that an OLP-specific PDGFRα regulatory element was originally presents in the common ancestors of fish and tetrapods, but was lost following separation of the two lineages. It is also possible that the element was lost in some but not all fish, perhaps being lost in small fish where the amount of C`NS myelin was not so critical for survival. Myelin would be expected to confer a selective advantage to large animals in which conduction velocity of action potentials could become limiting, for example motor control.

Chapter 6

Astroglia in Zebrafish and Xenopus

6.1 Introduction

In mammals, the origins and differentiation of oligodendrocytes are reasonably well characterised. This is in contrast to the current molecular understanding of the development and diversity of astrocytes; the most abundant cells of the CNS (see Chapter 1). Historically, astrocytes were perceived as a homogeneous population with limited functions, providing passive support for neurons. This view is changing with new data suggesting that they might modulate almost every aspect of functional neural networks (Scheffler et al., 2003). Furthermore, disregulation of astrocyte functions has also been implicated in pathogenesis of numerous developmental, genetic and acquired neurodegenerative diseases (Prat and Antel, 2005).

Similar to neurons and oligodendrocytes, astrocytes are thought to arise from multipotent neural stem cells in the neuroepithelium. New markers are emerging for the identification of astrocytes. Among others, Fibroblast Growth Factor Receptor 3 (FGFR3) and glutamine synthetase (GS) can now be identified at early stages in the chicken (E8) and mouse (E13.5) spinal cord neuroepithelium (Pringle et al., 2003). As with oligodendrocytes, FGFR3 and GS positive cells leave the neuroepithelium at later stages and distribute in the white and grey matter of the spinal cord. These cells are thought to be astrocytes precursors. Studies in mammals suggests that Olig2 inhibits astrocyte formation and that they do not normally develop from the Olig2-expressing pMN domain (Takebayashi et al., 2002; Zhou and Anderson, 2002).

The specific origins and development of astrocytes in higher vertebrates is not well defined (see Chapter 1) and even less is known about zebrafish and *Xenopus.*

Astrocytes and radial glia (identified by GFAP immunostaining) have been described primarily in the adult spinal cord of *Xenopus:* (Yoshida and Macklin, 2005) and in teleost fish (Kalman, 1998; Kruger and Maxwell, 1966), including zebrafish (Kawai et al., 2001; Tomizawa et al., 2000).

Although several subtypes of astrocytes exist in mouse, it is not clear whether different types of astrocytes exist in zebrafish and *Xenopus*. However, immunohistochemical evidence for distinct subtypes has been obtained using anti GFAP and other antibodies. Thus, in *Xenopus*, Yoshida and Colman (2001) describe GFAP and Vimentin in complementary domains. Similarly in zebrafish, Tomizawa et al. (2000) described C4 antibody positive cells, presumably astrocytes, that were distinct from GFAP-positive cells. However, there are not yet sufficient data to characterise astrocytes and little is known about how astrocytes are specified during development in zebrafish and *Xenopus*. Moreover, most studies describing astrocytes have been based on the use of antibodies that mainly label cell processes in the white matter, but not the cell bodies.

This chapter looks into the spatial and temporal gene expression patterns of the astrocytic markers Fgfr3, GS and Gfap in zebrafish and *Xenopus* spinal cord, from early development until adult stages. I examined their expression by *in situ* hybridization and immunohistochemistry. ESTs fragments for these markers were available to use as molecular probes as well as commercially available GFAP and GS antibodies (see Sections 2.4.1 and 2.5). Two zebrafish glutamine synthetase mRNAs (*gs01* and *gsn02*) are described here. These two *gs* genes exist due to the duplication of much of the zebrafish genome (Murray et al., 2003).

6.2 Results

6.2.1 *Fgfr3*, *gs* and *gfap* expression in zebrafish spinal cord

6.2.1.1 Pharyngula period

Forty eight hours postfertilization (48hpf). RNA *in situ* hybridization analysis in transverse sections across the trunk showed *fgfr3* expression in a band along the dorsal ventral VZ of the neural tube including the floor plate (Figure 6.1A). There was, however, a small region in the ventral VZ, which appeared less stained than the rest. A gap in FGFR3 expression has been previously described in the chicken VZ in a comparable position (Pringle et al., 2003). Glutamine synthetase 01 (*gs01*) expression was absent at this stage in the neural tube, although glutamine synthetase 02 *(gs02)* expression was present along the neural tube

(Figure 6.1B, C). GFAP immunoreactivity was widely distributed in very thin processes in the white matter adjacent to the pial surface of the spinal cord (Figure 6.1D), consistent with the findings of Marcus and Easter (1995).

6.2.1.2 Hatching period

Seventy two hours postfertilization (72hpf). By this stage *fgfr3* mRNA expression was observed in individual cells mainly in the intermediate or dorsal regions of the grey matter. Hardly any expression was found in the ventral region or within the white matter (Figure 6.1E). Zebrafish *gs01* expression was detected for the first time in individual cells in the grey matter, in a specific band of cells surrounding the central canal near the dorsal-ventral midline. The expression of *gs02* was detectable in single cells in the grey matter (Figure 6.1G). GFAP immunoreactivity was observed in thin radially orientated processes in the white matter, similar to the expression found at 48hpf. The GFAP-positive cell processes appeared somewhat thicker at this stage (Figure 6.1H).

I studied the expression of these markers at slightly later stages to look for any changes, in particular, whether individual cells distributed in white matter around the spinal cord, as has been reported in higher vertebrates (Collarini et al., 1991). At 96hpf, *fgrf3*, *gs01*, *gs02* expression was still observed in individual cells mainly in the intermediate region of the spinal cord. A few positive cells were sometimes observed in dorsal regions. A very similar pattern of expression was found among these three markers (Figure 6.1I-K). GFAP protein expression was present in radial processes that extended all the way from the central canal towards the pial surface of the spinal cord. The GFAP-containing glial processes appeared more prominent than in previous stages (Figure 6.1 L,O).

Double labelling with *gs01 in situ* hybridization and GS immunolabelling at 96hpf (Figure 6.1 M-O) showed co-expression of both markers in the same cells, indicating that GS antibody staining somehow mimics the expression of its mRNA in zebrafish. The expression of *gs01* mRNA was present mainly in cell bodies in the grey matter, although some processes appeared also to be stained while GS immunostaining was observed around the same cell bodies and in processes extending to the lateral pial surface.

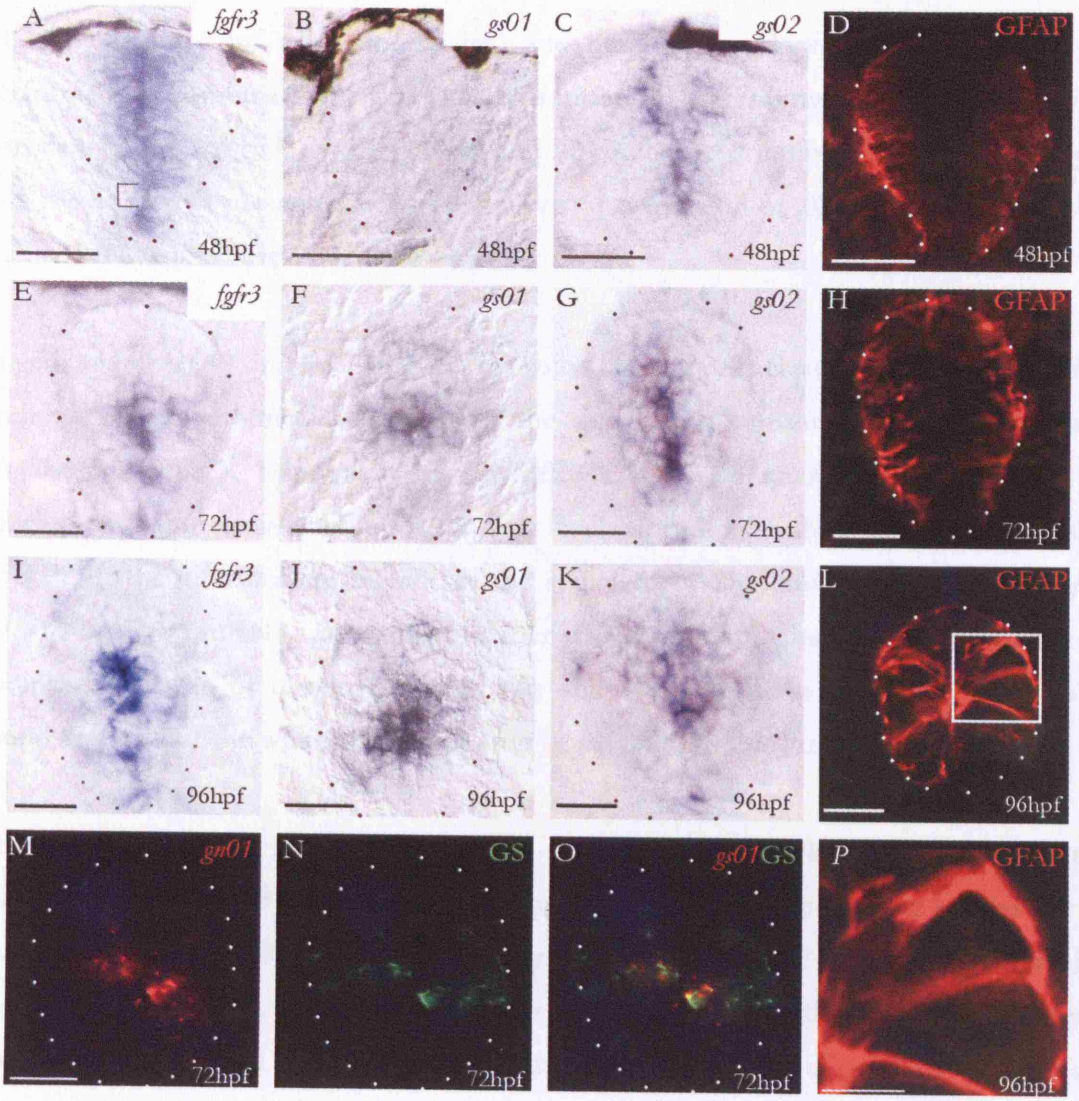


Figure 6. 1 Developmental expression of *fgfr3*, *gs01/02* and *gfap* in zebrafish spinal cord. Spinal cord transverse sections of 48hpf (**A-D**) 72hpf (**E-H**) and 96hpf **(I-L**) showing the specific location of *fgfr3* (**A,E,I**), *gs01* (**B,F,J**), *gs02* (**C,G,K**) and GFAP protein (red, IMF,**D,H,L**) within the spinal cord. GFAP higher magnification of L (**P**) Double immunolabelling with *gs01* mRNA (red,ISH,**M**) and GS protein (**N**,green, IMF) shows co-localization within the same cells (**M,N,O**). Scale bars: 20µm (A-O) 30µm (P)

6.2.1.3 Larval and young adult period

At later stages -7dpf (data not shown) and one-month-old zebrafish (Figure 6.2)- I performed a combination of *in situ* hybridization and immunohistochemistry with astrocytic markers: *fgfr3, gs01, gs02, gfap* and GS and GFAP antibodies. Although only a part of the data is presented here, the pattern of expression of all the astrocytic markers studied was very similar at all these stages.

In general, *fgfr3, gs01* and *gs02* mRNA expression were in cells clustered in the grey matter, preferentially in intermediate regions of the spinal cord surrounding the central canal (Figure 6.2 A,B,C). Expression of *gfap* mRNA was also studied at these stages of development and showed cell bodies in very similar locations to those expressing *fgfr3* and *gs01/02* in the intermediate regions of the spinal cord. The limited expression of *fgfr3, gs01/02* and *gfap* mRNAs in the grey matter is in contrast to that seen in the mouse at comparable stages of development. In this animal the expression of these markers is found throughout the white and grey matter of the spinal cord (Pringle et al 2003).

The restricted mRNA staining locations in zebrafish was remarkably similar for all the markers, however f*gfr3* and *gs02* staining in the grey matter appeared to be broader in extent than that of *gs01* or *gfap* mRNA expression. Zebrafish *gs02* mRNA expression also showed a difference in staining compared to *gs01* and the rest of the markers; that is, *gs02* staining was in a small number of cells in the white matter and close to the pial surface (Figure 6.2 C). These differences in expression were hard to appreciate in the spinal cord data presented here, but easier in more rostral levels of the CNS (such as hindbrain and telencephalon) where *gs*02 was expressed close to the pial surface and *gs01* was absent (data not shown). GFAP immunoreactivity was strongly expressed in radially orientated cell processes extending from the central canal all the way to the pial surface. The fibres also appeared to thicken towards the pial surface.

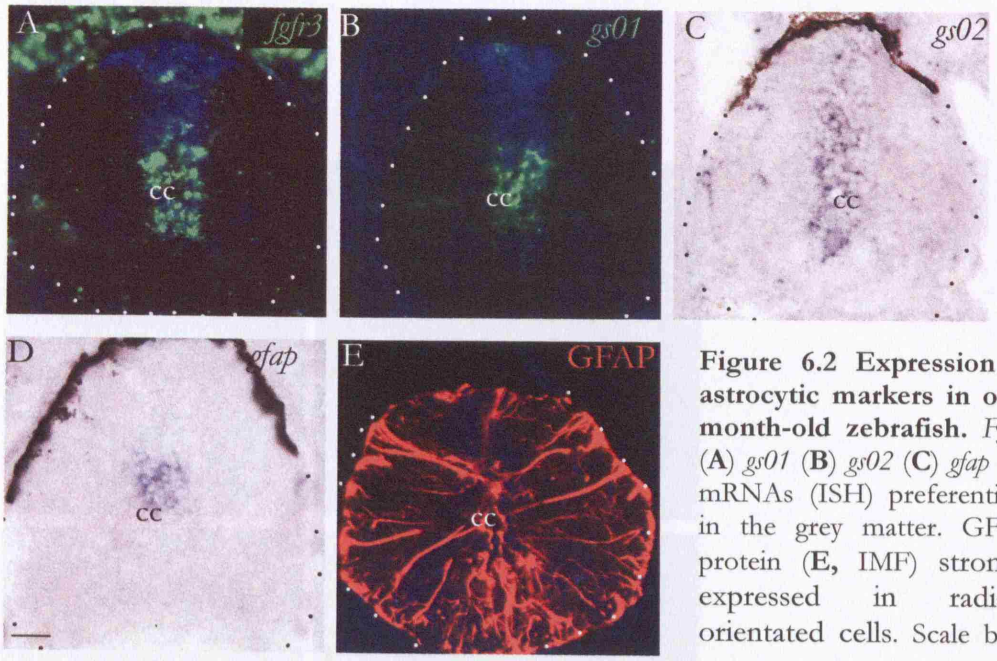


Figure 6.2 Expression of astrocytic markers in one-month-old zebrafish. *Fgfr3* (**A**) *gs01* (**B**) *gs02* (**C**) *gfap* (**D**) mRNAs (ISH) preferentially in the grey matter. GFAP protein (**E,** IMF) strongly expressed in radially orientated cells. Scale bars:

I performed double immunolabelling with GS and GFAP proteins of 7dpf and one-month old zebrafish spinal cord to investigate whether the two proteins were expressed in similar cells or processes (Figure 6.3). GFAP immunolabelling was present in radiating processes, symmetrically distributed. In contrast, GS protein expression appeared in a more restricted distribution, mainly in filaments extending laterally and only a very few, thin processes towards dorsal or ventral positions. However, the expression of both proteins showed overlap in some cells around the central canal and many of the filaments in the white matter and the pial surface end feet. Many cells around the central canal and processes were stained GFAP positive, although in some cases they were also GS negative and vice versa. These patterns of expression were preserved at all stages examined.

Triple labelling studies with *gs01* or *gs02* mRNA together with GS and GFAP antibodies showed that *gs01* and *gs02* mRNA positive cells, localized in the grey matter, occupied the

same region from which the GS and GFAP cell bodies extended. Some of the GFAP and GS protein expression co-localized with some of the *gs01/02* mRNA positive cells (Figure 6.4). Furthermore, *gs01* mRNA expression co-localized to a greater degree with the GS immunostaining than with *gs02*.

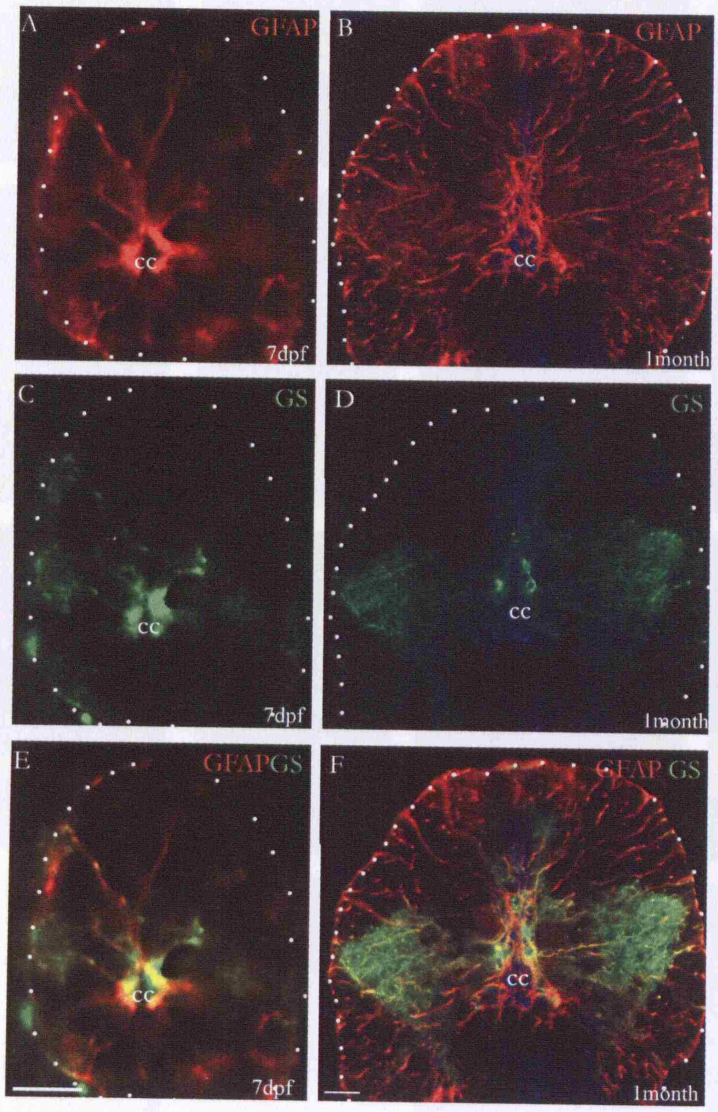


Figure 6.3 GFAP and GS double labelling in zebrafish spinal cord. GFAP (red, IMF, **A,B**) and GS (green, IMF, **C,D**) and double labelling (**E,F**) expression at 7dpf and one-month-old. Sections were counter stained with DAPI to localize all nuclei (blue). Scale bars: 20μm.

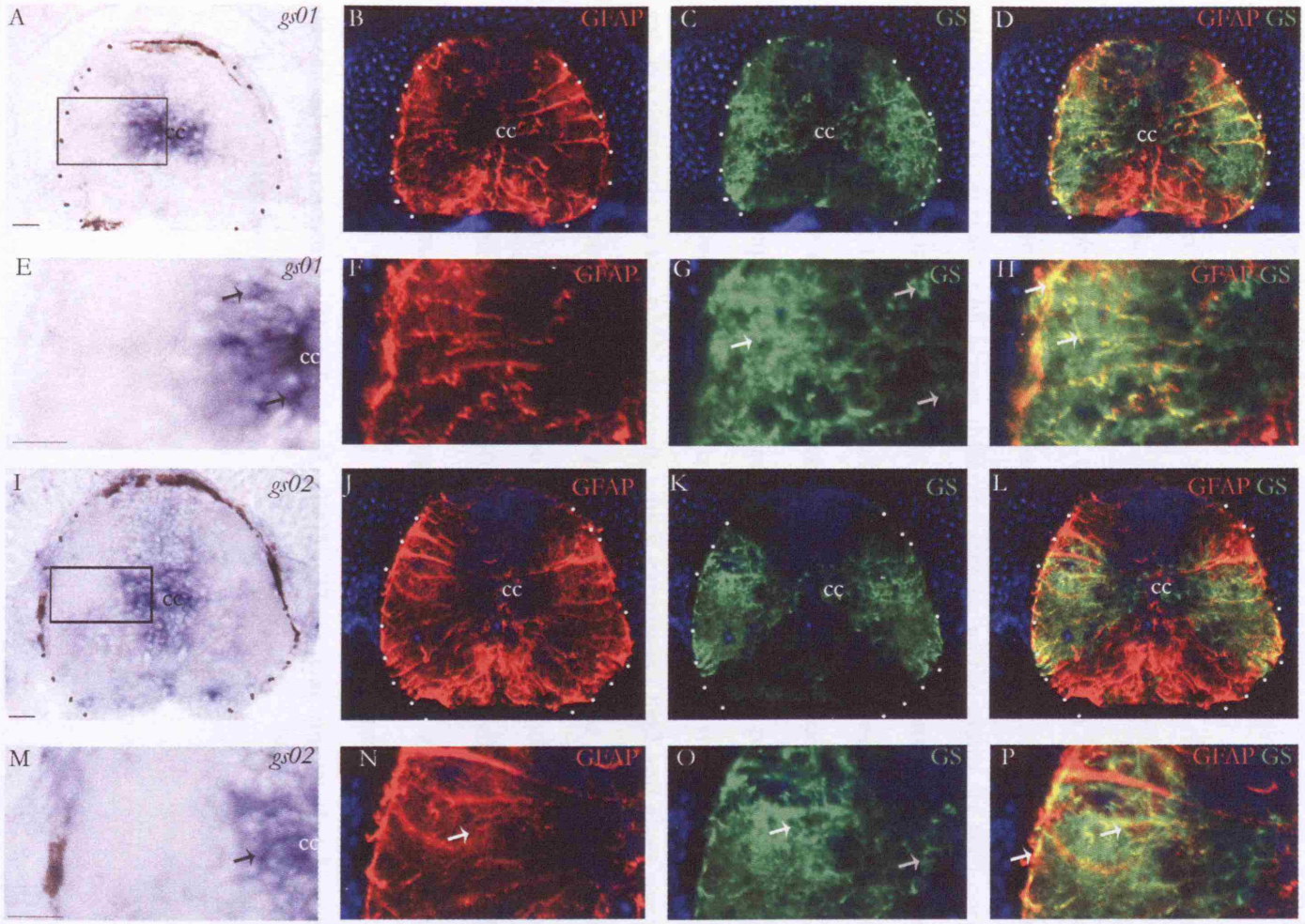


Figure 6.4 *Gs* **mRNA and GS and GFAP protein expression in one-month-old zebrafish spinal cord.** Transverse spinal cord sections were triple stained with either *gs01*(**A, E**) or *gs02* (**I,L**) together with GFAP (**B,F,J,N**) and GS (**C,G,K,O**) antibodies. *Gs01* and *gs02* mRNA positive cells, localised in the grey matter occupying the same region from which the GS and GFAP cell bodies extended. Double labelling with GFAP and GS (**D,H,L,P**) showed co-localization within the same process and end feet of the pial surface (arrows). Scale bars: 20µm.

6.2.2 FGFR3, GS and GFAP expression in *Xenopus* spinal cord

6.2.2.1 Early development

Xenopus spinal cord sections were fixed and processed for *in situ* hybridization or immunohistochemistry to examine the expression of Fgfr3, GS and GFAP markers. Specific *Xenopus Fgfr3* and *GS* cDNA were acquired (Section 2.4.1). Antibodies against GS and GFAP were used in the same manner as described for zebrafish (Section 2.5). These antibodies cross react well across different species, as the genes are well conserved. *Fgfr3* mRNA expression in *Xenopus* at early stage (stage 40) (Figure 6.5A) was observed in the neuroepithelium of the spinal cord, with a very similar expression pattern to that in chick and mouse (Pringle et al., 2003) as well as zebrafish spinal cord (Figure 6.1). At a similar stage, *GS* mRNA expression was present with a very similar pattern to *Fgfr3* (Figure 6.5B). A small gap was observed in the ventral region of the *Fgfr3* staining pattern. In chick, this gap corresponds to the pMN domain, which expresses Olig2 and gives rise to OLPs and motor neurons. I performed double *in situ* hybridization for *Fgfr3* with *Pdgfrα* and *Sox10* that, as described in Chapter 5, define the origin of oligodendrocytes in the ventral region of the *Xenopus* spinal cord. At stage 40, *Fgfr3* presented a small gap between the floor plate and higher domains. Double labelling showed that Sox10 and *Pdgfrα* (data not shown) mRNA expression were within the space of *Fgfr3* expression (Figure 6.5 E-G), as described in mammals (Pringle et al., 2003). There was no overlap between the *Fgfr3* signal and either *Sox10* or *Pdgfrα*.

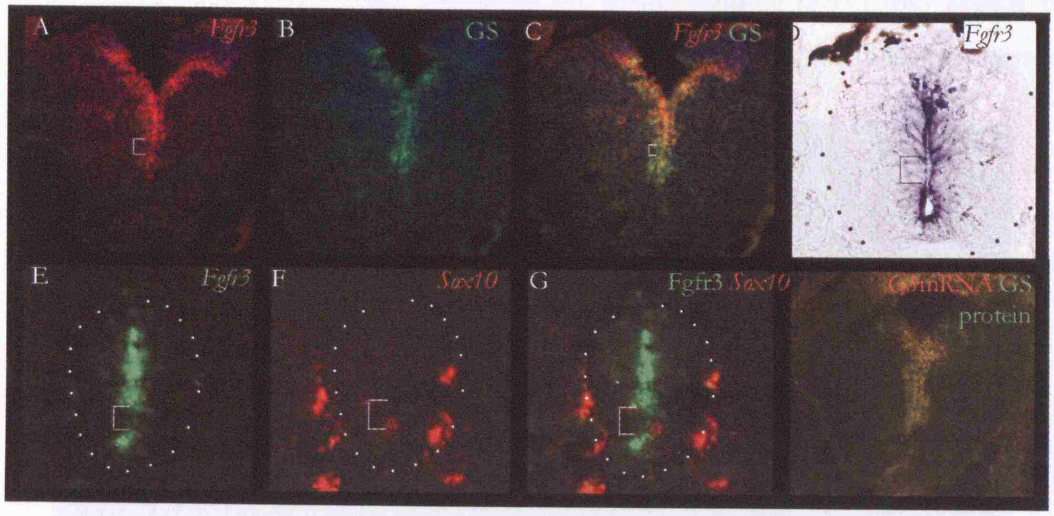


Figure 6.5 Expression of astrocytic markers in early *Xenopus* spinal cord. Spinal cord sections of stage 40 in *Xenopus* showing the specific location of *Fgfr3* (**A**), GS (**B**) and double labelling (**C**) within the spinal cord. Double *in situ* hybridization showed a significant overlap (yellow) between *Fgfr3* (red) and GS (green) (ISH). *Fgfr3* mRNA expression in a more dorsal spinal cord section (**D**). Double *in situ* hybridization for Fgfr3 and *Sox10* (**E-G**) (ISH). There is no significant overlap between the *Fgfr3* (green) signal and *Sox10* (red).

6.2.2.2 Adult *Xenopus*

Astrocyte lineage markers Fgfr3 and GS were studied at later stages in *Xenopus*. At six-month-old Fgfr3 and Gs mRNA expression was confined to the neuroepithelial (ependymal) cells around the central canal of the spinal cord (Figure 6.6A, B)

GFAP and GS immunolabelling in six-month old *Xenopus* spinal cord showed GFAP expression in processes radially extending from the central canal towards the periphery, establishing contacts with the end feet of the pial surface (Figure 6.6C). Stronger expression was mainly seen in the white matter rather than in the grey matter. GS expression was also observed in filaments extending from the central canal towards the pial surface (Figure 6.6D). GFAP and GS stained fibres appeared to thicken towards the glial end feet. Double immunolabelling showed overlap of GFAP and GS labelling in most of the processes near the pial surface (Figure 6.6F-J). GS protein was also observed in some cell bodies in the white matter across the spinal cord. Most of the cell bodies were in the white matter and this cell body staining did not co-localize with astrocytic

marker GFAP. However, as they expressed the nuclei staining DAPI they appear to be the cell bodies of an unknown cell type, perhaps GFAP-negative "fibroblast astrocytes".

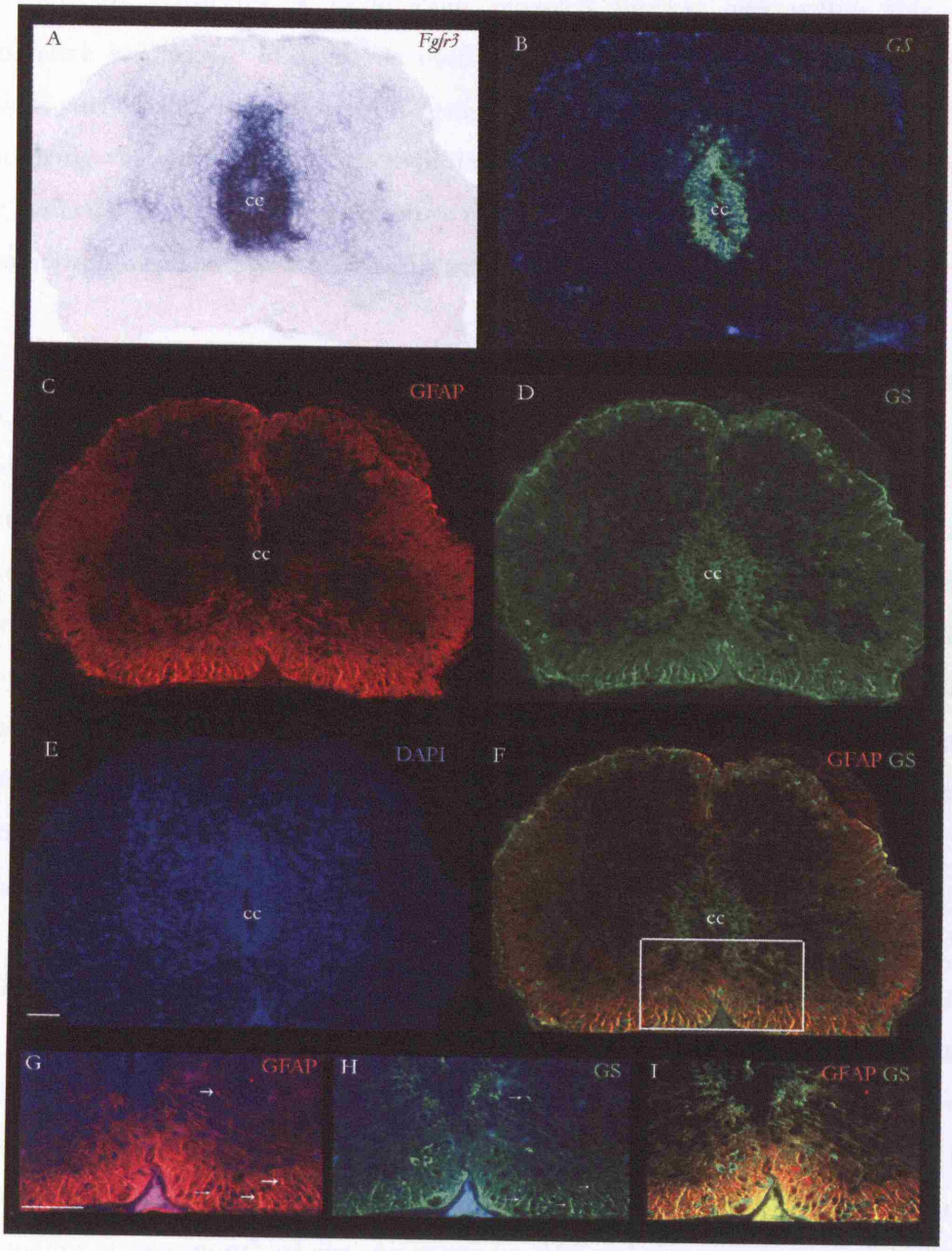


Figure 6.6 Expression of astrocytic markers in adult *Xenopus* spinal cord. Transverse sections through six-months-old spinal cord showing the specific location of *Fgfr3* (blue, ISH, **A**) and *Gs* (green, ISH, **B**) mRNA. Their expression was localised surrounding the central canal (cc). GFAP (red, IMF **C**) and GS (green,IMF, **D**) protein double labelling in processes from the central canal towards the endfeet (**C**). Most of the processes overlap between both proteins (**F**). Sections were counterstained with DAPI to localize all nuclei (blue,**E**). Higher magnification from D **(G-I)**. Scale bars: 20µm.

6.3 Discussion

The study of astrocytes is undergoing renewed interest and within this context comparative studies are likely to be useful in clarifying their complex functions. This Thesis is part of an ongoing line of investigation to establish zebrafish as a model system for studying vertebrate oligodendrocyte development specifically, and glial development more generally. It seems natural therefore to expand this work to include astrocytes. Little information is available about these cells in zebrafish.

In this chapter I have described the expression patterns of astrocytic markers Fgfr3, Gs and Gfap by mRNA expression (permitting the detection of cell bodies) and protein expression by GS and GFAP (labelling glial processes), in embryonic and adult stages of zebrafish and, in parallel, *Xenopus* spinal cord. These markers have been used to characterize astrocytes in chick and mouse spinal cord (Pringle et al., 2003). In general, my studies in zebrafish and *Xenopus* described astrocyte cell bodies mainly in the VZ and nearby grey matter with processes that extended radially to the pial surface. In addition, the intensity of GFAP and GS immunolabelling was higher in glial processes within white matter than in grey matter. This is consistent with the general description of astrocytes and radial glial in adult zebrafish and other teleost, such as carp and trout as well as X*enopus* by immunostaining studies mainly with GFAP (Miller and Liuzzi, 1986).

In zebrafish at 48hpf, when oligodendrocytes are being produced in the ventral spinal cord, *fgfr3* and *gs02* mRNA expression were found in a band of cells in the VZ near the dorsal-ventral midline. At later stages, *fgfr3* and *gs02,* together with *gs01* and *gfap* mRNA expression, were markedly similar in their localization in individual cells, mainly in cells around the central canal. A very small number of *gs02* expressing cells were seen in the white matter at one-month of age. As in mouse *fgfr3* and *gs* expression was initially found along the VZ. However, in mouse at later stages Fgfr3 and Gs positive cells leave this region and spread throughout the white and grey matter of the spinal cord (Pringle et al., 2003).

In *Xenopus*, at the time of oligodendrocyte production characterized in chapter 5 (stage 40), Fgfr3 and GS transcripts co-localized to the same region of the VZ in the

intermediate spinal cord. A similar distribution of these markers has been described in mouse and chicken spinal cord at comparable stages of development (Pringle et al., 2003).

In adult *Xenopus* spinal cord, Fgfr3 and Gs mRNA expression were confined to cell bodies surrounding the central canal of the spinal cord. This is different to the mouse, in which FGFR3 and GS is expressed in individual cells throughout the white and grey matter (Pringle et al., 2003). The location of astrocytes cells bodies around the central canal in *Xenopus* has previously been described by Miller and Liuzzi (1986). They described GFAP expression in glial processes transversing the white matter and by electron microscopy confirmed that that there were no astrocyte cell bodies either at the pial surface or throughout the white matter of the cord.

GFAP protein has been most frequently used as an astrocytic marker in lower vertebrates, including zebrafish and *Xenopus*, and higher vertebrates, including humans. Studies in mammals and chicken have also shown that GS protein labels astrocytes (Norenberg and Martinez-Hernandez, 1979; Norenberg, 1979) (Didier et al., 1986; Suarez et al., 1996; Tholey et al., 1987). Futhermore, GS has also been described in ependymal cells and astroglial cells in lizards (Monzon-Mayor et al., 1990) and carp (Kalman, 1998; Arochena et al., 2004).

Here, I first detected GFAP immuolabelling around the white matter of 48hpf zebrafish spinal cord, covering mainly the astrocyte endfeet, as described by Marcus and Easter (1995). Later in development, the intensity of GFAP labelling increased progressively in glial processes. Gs labelling was first observed at 72hpf and in very characteristic pattern; that is, radially from the central canal towards the lateral parts of the pial surface.

GFAP and GS immunolabelling in adult zebrafish and *Xenopus*, showed a general expression mainly in glial processes orientated from the central canal to the pial surface. A higher intensity of both antibodies was seen in white matter rather than in the grey matter of the spinal cord in both species. GFAP and GS shared a very similar pattern in *Xenopus*. GS expression in zebrafish, however, was preferentially orientated laterally. Most of the glial processes and end feet at the pial surface contained both proteins.

Moreover, the cell bodies of the filaments stained by GFAP and GS proteins were in the grey matter of spinal cord at the same location than *gs01/02*, *fgfr3* and *gfap* transcripts in zebrafish identified by the triple labelling (Figure 6.6). In general, GFAP and GS immunoreactivity showed a very similar distribution pattern in the adult and larval spinal cord.

GS antibody also labelled the cell bodies of some unidentified cells, mainly in the white matter of *Xenopus*. A similar type of staining has been described in rainbow trout spinal cord, with microtubule associated protein (MAP1B) present in neurons and a glial subpopulation. Glial processes stained by MAP1B co-localized with GFAP within the processes in the VZ of the spinal cord and in the pial surface (Alfei et al., 2004). From their location along the fibres it is possible that these cells are radial glial cell bodies as, unlike mammals, lower vertebrates such as fish retain most of their radial astroglia throughout life (Rubio et al., 1992; Kalman, 1998). Further experiments are necessary to characterize these cells with radial glial markers (for example, RC2) or neuronal markers.

Stereotypical, stellate-shaped astrocytes were not found in any of the zebrafish or *Xenopus* spinal cord sections with GFAP or GS protein staining. This is consistent with other studies based on GFAP immunostaining in fish or other teleosts (Cyprinus carpio, atlantic hagfish) which have also been unable to identify stellate astrocytes in the brain or spinal cord (Rubio et al., 1992; Mugnaini and Walberg, 1965; Lara et al., 1989). Of course, stellate astrocytes are a common observation in mammalian and avian CNS.

Two different glutamine synthetase genes, *gs01/02*, have been described in zebrafish, trout and toad fish (Murray et al., 2003). This is due to a duplication of the teleost lineage genome (Amores et al., 1998; Postlethwait et al., 1998). GS01/02 have not been characterised in zebrafish before, however in trout and toad fish where it is also duplicated, there are different expression patterns outside the spinal cord; for example, *gs02* gene is mainly expressed in the gills (Murray et al., 2003). The expressions of *gs01* and *gs02* in spinal cord seen in this study were slightly different. The expression of *gs02* started earlier with a broader expression, meanwhile *gs01* started later and had a more constrained expression around the central canal. At later stages, *gs02* was observed in some individual cells in the white matter of the spinal cord and in the end feet of the pial surface, being

more obvious in more rostral regions. An explanation of the differential expression comes from the assumption that they may have different functions. The expression of *gs01* had a closer pattern to *gfap* mRNA, which is seen in astroglia. In contrast, *gs02* was expressed in both astroglia and other types of cells. In addition, from the triple staining the GS protein had a closer expression pattern to the *gs01* mRNA. Different expressions have been described for *gs01/gs02* outside the CNS in other species (Walsh et al., 2003). Toad fish *gs02* is highly expressed in the gills in comparison to the expression of *gs01*, which is expressed in several regions, including (in descending order of expression): brain, liver, stomach, kidney, intestine and gills. It is possible that the duplicated genes *gs01/gs02* in zebrafish are expressed in different tissues in the CNS. Whether both genes are expressed in different types of astrocytes or other type of cells would be an interesting future study, but it has not been investigated here.

Chapter 7

General Discussion

The series of studies described in this thesis have characterized the development and differentiation of glial cells, primarily oligodendrocytes, in zebrafish spinal cord. Comparative studies with other models, mouse and *Xenopus*, suggest that oligodendrocyte production, proliferation and myelin generation are generally conserved in fish. I conclude that zebrafish is a suitable model to study oligodendrocyte development in vertebrates.

7.1 Oligodendrocyte specification and differentiation in zebrafish spinal cord

Most studies of neurogenesis and gliogenesis (including oligodendrocyte development) have considered the well characterized and relatively simple patterning of the mouse spinal cord (Briscoe et al., 2000; Briscoe and Ericson, 1999; Jessell, 2000). The zebrafish spinal cord is beginning to be as well characterised as that of the mouse. My work and other published studies show that at early stages the zebrafish spinal cord develops very similarly to that of mammals (Park et al., 2004; Park et al., 2002; Shin et al., 2003). The expression of neural precursors markers such as *iro3, pax6, olig2, nk2.2* in fish closely resembles their pattern of expression in mouse (Barth and Wilson, 1995; Tan et al., 1999). This new understanding of the zebrafish spinal cord patterning provides the essential groundwork for future studies.

In general zebrafish spinal cord OLPs have a similar ventral region of origin to that of most OLPs in mammals and go through similar stages of development prior to myelination. Zebrafish OLPs, identified by *olig1, olig2* and *sox10* markers, appear in the ventral neural tube dorsal to the *nk2.2*-expressing domain and proliferate while migrating throughout the spinal cord. Eventually they start expressing myelin protein genes (*mbp, plp* and p_0) and change morphology from a simple bipolar form to cells with multiple processes. In zebrafish adult spinal cord, a significant proportion of cells show a lack of co-localization between OLP specific markers and the myelin marker *mbp*. These cells could represent immature progenitors as seen in mammals (Watanabe et al., 2002; Watanabe et al., 2004; Levison et al., 1999). Figure 1 shows a schematic representation of oligodendrocyte development in mouse and zebrafish spinal cord compared at different developmental stages.

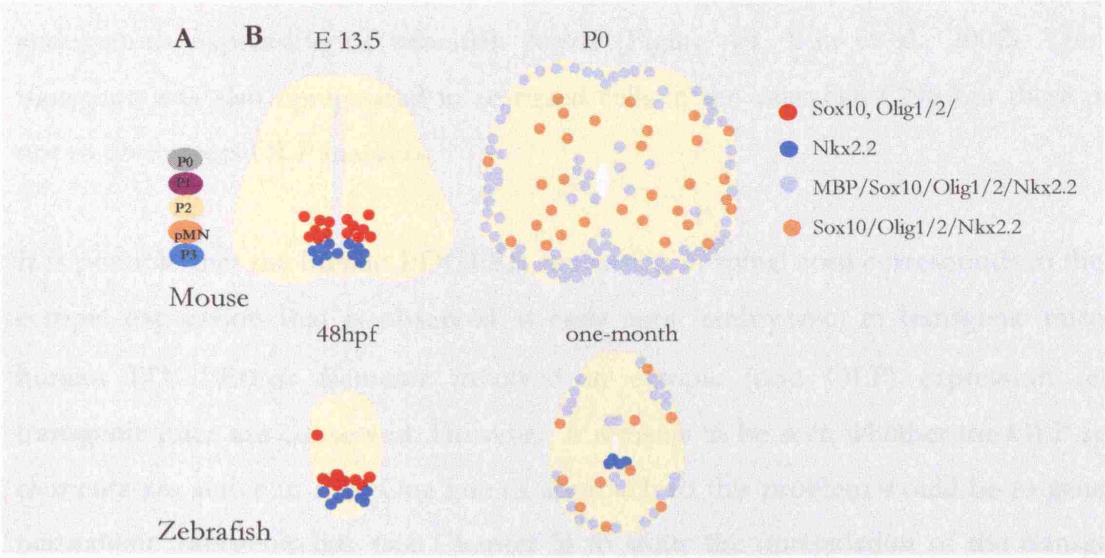


Figure 7.1 Oligodendrocyte development in mouse spinal cord at different developmental stages in comparison to zebrafish. A. Progenitor domains in the ventral spinal cord, p3, pMN, p2, p and p0. **B** Nkx2.2 expression (shown in dark blue dots) is ventral to the Olig1/Olig2/Sox10/Pdgfrα region of expression (shown in red) at E13.5 in mouse and 48hpf zebrafish. At postnatal stages, precursor cells become mature oligodendrocytes expressing myelin proteins (shown in light blue), localised mainly in the white matter. A proportion of Sox10, Olig2 and Nk2.2 cells lack the myelin marker MBP. A cluster of zebrafish *nk2.2* expressing cells persist in the ventral region in zebrafish in comparison to same gene in mammals, where it is distributed throughout the spinal cord.

7.2 Human PDGFRα regulation in zebrafish

Overall, the majority of the genes involved in oligodendrocyte development studied here show similar expression characteristics to their homologues in mouse, however there are also some differences.

Pdgfrα, a specific, well-defined and characterised OLP marker in *Xenopus*, mouse and human is not expressed in zebrafish OLPs. Zebrafish *pdgfrα* expression does however mimic that of higher vertebrates in tissues outside the CNS such as mesenchyme and neural crest (Liu et al., 2002).

Transient expression of a human PDGFRα 110kb BAC transgene containing both non-CNS and OLPs specific cis-regulatory elements gives expression of the human PDGFRα transgene in non-CNS tissues (mesenchyme and neural crest) that mimics the endogenous expression of zebrafish *pdgfrα* (Figure 4.1, (Liu et al., 2002). The BAC transgene was also upregulated in scattered cells in the zebrafish CNS but these proved not to co-express OLP markers.

It is possible that the human PDGFRα expression in spinal cord corresponds to the same ectopic expression that is observed at early ages (embryonic) in transgenic mice. The human PDGFRα *cis* elements involved in ectopic (non OLP) expression seen in transgenic mice are conserved. However, it remains to be seen whether the OLP specific elements are active in fish. One line of approach to this problem would be to generate a permanent transgenic line (see Chapter 5) to study the upregulation of the transgene in OLPs at later stages. Such a study is not possible using the transient transgenic approach.

7.3 Olig1 expression in zebrafish

Another difference between oligodendrocyte development in zebrafish and mice is in *olig1* expression. In mouse, Olig1 is co-expressed with Olig2 throughout development. In zebrafish however, *olig1* expression appears later than *olig2* in the neuroepithelium of the neural tube. Both, *olig1* and *olig2* mRNAs were seen co-expressed in OLPs, however *olig1* was down regulated in differentiating oligodendrocytes, an inference that can be made as no co-localisation was observed between *olig1* and the zebrafish myelin marker *mbp*. In mammals, Arnett et al, (2004) found that Olig1 protein is expressed in differentiated oligodendrocytes, but translocated to the cytoplasm of these cells compared to Olig2 protein, which persist in the nucleus. In addition, these authors found Olig1 protein in the nucleus of demyelinated regions in mice and post mortem tissue from MS patients. This shows a possible role of Olig1 in remyelination, re-appearing in regenerating precursors and its requirement for myelin regeneration. It would be very interesting to study zebrafish *olig1* expression in normal, damaged and regenerated tissue to identify whether there is a difference between *olig1* and *olig2* expression in these zebrafish tissues.

One of the major advantages of zebrafish as a model organism is the ability to perform large-scale mutant screens. A large number of neurological mutants have been identified in detail (Driever et al., 1996; Schier et al., 1996). In this work, a preliminary screening of neurological mutants was begun by Kazakova et al, (2004), searching for defects in myelination in zebrafish. The CNS and PNS myelin marker *mbp* was used to screen 45 mutants held in the UCL fish facility. Among them *otter* mutant showed a lack of *mbp* in the lateral line (PNS) (Kazakova et al., 2004). I further characterised myelin and early oligodendrocyte specification in the CNS in the *otter* mutant. This showed a lack of *mbp* and *plp*, but the presence of a small number of p_0 cells. There was also a delay in the specification of oligodendrocytes. The identification of this gene is currently underway in another laboratory (Guo, S Department Neuroscience, California, USA). Other myelin mutants have already been identified in the screen (Kazakova et al., 2004). Amongst those identified is the *Raldh2* mutant, with defects in *mbp* expression in Schwann cells of the PLL and in a subset of oligodendrocytes in the hindbrain (Kazakova et al., 2004). Similar to the *otter* mutant, myelin and early oligodendrocyte characterization in the CNS will be fundamental. The identification of new genes involved in the oligodendrocyte lineage specification has improved our understanding of the signalling requirements underlying the generation of oligodendrocytes from neuroepithelial or stem cells.

7.4 Astrocyte characterization

Astrocytes are the most abundant cells within the CNS and are involved in many functions (Scheffler et al., 2003), but are still poorly characterized. How many different subtypes exist, what functions they perform, their specification and the signalling mechanisms involved are some of the many questions still unresolved.

Astrocytic markers have already been characterized in mouse spinal cord (Pringle et al., 2003). Zebrafish *fgfr3*, *gs* and *gfap*, were also expressed in the spinal cord (Chapter 6). Both GS and GFAP proteins had a radial distribution, extending from the central canal towards the glial end feet at the pial surface. Meanwhile, cell bodies detected by mRNA expression of these markers were mainly clustered in the grey matter around the central canal. This differs from mouse in which the same markers are located throughout the grey and white matter at late stages (Pringle et al., 2003).

7.5 Future directions

This work forms the first step in an experimental programme designed to find new genes in the development of the oligodendrocyte pathway.

One of the main advantages of zebrafish is the transparency of embryos at early stage. This allows cell development to be visualized in real time. One approach already taken in Richardson/Smith lab and other labs is the generation of transgenic fish lines where oligodendrocyte can be labelled with fluorescent markers (GFP of RFP) marking different stages in development and differentiation of oligodendrocytes. A number of PAC transgenic lines have been generated in Richardson/Smith lab and elsewhere driven by the regulatory sequences of *sox10*, *nk2.2*, *olig2*, *plp* (Shin et al., 2003; Yoshida and Macklin, 2005). These transgenic lines are very useful to study oligodendrocyte development in real time with the possibility of using time-lapse microscopy and setting up a mutant screen for mutant phenotypes looking for new genes regulating glial cell development.

Another advantage of zebrafish is the ease with which small molecules agonists/antagonists can be administered simply by adding the drugs to their tank water. This makes zebrafish a useful model for drug screening and the study of signalling pathways –particularly, in the present context those involved in the development of oligodendrocytes. It is known that Shh is essential for the specification of oligodendrocytes and recently it has been found that fibroblast growth factor (Fgf) can also induce oligodendrocyte progenitors. Using small molecules inhibitors of the hedgehog and Fgf2 signalling pathways, already tested in other studies (Park et al., 2002; Masai et al., 2003), it will be possible to further study the relationship between Shh and Fgf signalling as well as other signalling pathways such as retinoic acid, notch signalling and MAP-kinase, all of which have an effect in oligodendrocyte development in mouse (Kessaris et al., 2004; Chandran et al., 2003).

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